

44689

National Library
of CanadaBibliothèque nationale
du Canada

Canadian Theses Division

Division des thèses canadiennes

Ottawa, Canada
K1A 0N4**PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER**

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

PIARA SINGH BAINS

Date of Birth — Date de naissance

06-06-1948

Country of Birth — Lieu de naissance

INDIA

Permanent Address — Résidence fixe

2B, CLAREVIEW VILLAGE
EDMONTON, ALTA, T5A 3P2

Title of Thesis — Titre de la thèse

Pea Leaf mesophyll protoplasts: Isolation and inoculation
with clover yellow mosaic virus.

University — Université

University of Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

MSc

Year this degree conferred — Année d'obtention de ce grade

1980

Name of Supervisor — Nom du directeur de thèse

Dr. C. Hiruki

Permission is hereby granted to the NATIONAL LIBRARY OF
CANADA to microfilm this thesis and to lend or sell copies of
the film.The author reserves other publication rights, and neither the
thesis nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.L'autorisation est, par la présente, accordée à la BIBLIOTHÈ-
QUE NATIONALE DU CANADA de microfilmer cette thèse et de
prêter ou de vendre des exemplaires du film.L'auteur se réserve les autres droits de publication; ni la thèse
ni de longs extraits de celle-ci ne doivent être imprimés ou
autrement reproduits sans l'autorisation écrite de l'auteur.

Date

April 1, 1980

Signature

P. S. Bains



National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

THE UNIVERSITY OF ALBERTA

PEA LEAF MESOPHYLL PROTOPLASTS: ISOLATION
AND INFECTION WITH CLOVER YELLOW MOSAIC VIRUS

by



PIARA SINGH BAINS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE
IN
PLANT PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

SPRING 1980

THE UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies and Research, for acceptance, a
thesis entitled Pea Leaf Mesophyll Protoplasts: Isolation and
Infection with Clover Yellow Mosaic Virus submitted by Piara Singh
Bains in partial fulfilment of the requirements for the degree of
Master of Science in Plant Pathology.

..... C. Hiuiki

Supervisor

..... Fred S. Cook

..... W. P. Skaropad

.....

Date March 25, 1980.

ABSTRACT

Pea mesophyll protoplasts were isolated from fully expanded leaves of three-week-old 'Alaska' pea (Pisum sativum) plants, grown at 23°C with a daily light period of 14 hours at approximately 8,000 lux and at 21°C with a dark period of 10 hours. Both the 'one-step' and the 'modified two-step' methods that were employed for protoplasts isolation resulted in similar yields ($1-1.5 \times 10^7$ protoplasts per gram fresh weight of leaves). Moreover, the use of the enzyme Pectolyase Y23 in the digestion medium considerably reduced the time of protoplast isolation from two hours to 35 minutes.

The protoplasts were successfully inoculated with clover yellow mosaic virus (CYMV) by briefly incubating with a CYMV/poly-L-ornithine (PLO) mixture. The infection and the extent of CYMV multiplication were determined by infectivity assay of the protoplast extracts and fluorescent antibody staining of the inoculated protoplasts.

In the investigation of the effects of certain factors on infection of the pea mesophyll protoplasts with CYMV, PLO was essential for infection. The optimal concentrations of PLO and CYMV for a maximum infection were 1.0 to 1.4 µg/ml and 3 µg/ml, respectively. Since the percentage of protoplasts damaged was higher at the PLO concentration of 1.4 µg/ml than at 1.0 µg/ml, the latter concentration was used routinely in this investigation. Phosphate, when used as an inoculation buffer, resulted in a higher percentage of infection of protoplasts than did citrate buffer, and a pH of 6.3 or 0.025

M-phosphate buffer was optimal for infection. A 10-minute-incubation of protoplasts with the inoculum gave a relatively good infection rate, whereas different methods of inoculation had little effect on the rate of infection.

Under the conditions summarized above, infections of the protoplasts up to 57 percent were achieved as determined by the fluorescent antibody technique. The time-course study showed a synchronous virus infection and multiplication, and extensive fluorescence observed in the protoplasts indicated active virus multiplication.

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to Professor C. Hiruki for the valuable guidance, kind help and encouragement during the course of my research programme, and during the preparation of this manuscript.

I would also like to thank Dr. W. P. Skoropad for valuable suggestions at different phases of this investigation, for reviewing the manuscript and for serving on the thesis committee.

I wish to thank Dr. F. D. Cook, who served on the thesis committee and reviewed the manuscript.

I would like to express my appreciation to my colleagues, Drs. D. V. Rao, M. H. Chen, and T. Okuno for their help during this investigation.

The excellent technical assistance of Mr. T. Tribe, and assistance of Mr. H. Welling in growing plants is gratefully acknowledged.

I also extend my thanks to Mrs. Hardip Mann and Mrs. Martha Laverty for their excellent typing.

Financial assistance provided by the University of Alberta in the form of a Graduate Research Assistantship, and a Research Assistantship through an operating grant (A3843) from the National Science and Engineering Research Council of Canada to Dr. C. Hiruki is gratefully acknowledged.

Finally, I wish to thank my wife, Livleen, for her patience and concern in my studies, and Atwals for their encouragement and help during these years.

TABLE OF CONTENTS

<u>CHAPTER</u>		<u>Page</u>
1	INTRODUCTION	1
2	LITERATURE REVIEW.	4
	2.1 Isolation of Protoplasts.	4
	2.2 Inoculation and Incubation of Protoplasts	8
	2.3 Factors Affecting Infection of Protoplasts.	11
	2.4 Detection of Virus Infection and Multi- plication in Protoplasts.	17
3	MATERIAL AND METHODS	23
	3.1 Isolation of Pea Mesophyll Protoplasts.	23
	A. Source plant.	23
	B. Isolation of protoplasts.	23
	3.2 Virus	24
	3.3 Inoculation of Protoplasts.	25
	3.4 Infectivity Assay	26
	3.5 Fluorescent Antibody Preparation.	27
	A. Production of immunoglobulin.	27
	B. Conjugation with fluorescein iso- thiocyanate	27
	3.6 Staining of Protoplasts with Fluorescent Antibody.	28
	3.7 Scanning Electron Microscopy.	28
4	RESULTS.	29
	4.1 Method of Isolation	29
	4.2 pH of Inoculum.	29
	4.3 Kind of Buffer.	33
	4.4 Concentration of Poly-L-ornithine	33
	4.5 Method of Inoculation	33

<u>CHAPTER</u>	<u>Page</u>
4.6 Concentration of Inoculum	36
4.7 Duration of Inoculation	36
4.8 Multiplication of Clover Yellow Mosaic Virus	41
5 DISCUSSION	46
6 PLATES AND LEGENDS	52
BIBLIOGRAPHY	57

LIST OF TABLES

DESCRIPTION

<u>Table</u>		<u>Page</u>
1.	Comparison of different methods used for isolation of pea leaf mesophyll protoplasts	30
2.	The time-course of clover yellow mosaic virus multiplication in pea mesophyll protoplasts as determined by fluorescent antibody technique	44

LIST OF FIGURES

DESCRIPTION

<u>Figure</u>		<u>Page</u>
1.	Effect of pH of inoculation buffer on clover yellow mosaic virus (CYMV) infection of pea mesophyll protoplasts. The protoplasts were inoculated with 5 µg per ml CYMV in the presence of 1 µg per ml poly-L-ornithine. The protoplasts were stained with fluorescent antibody specific to CYMV after 44 hours of incubation.	32
2.	Effect of poly-L-ornithine concentrations in the inoculum on survival of pea mesophyll protoplasts and their infection with clover yellow mosaic virus (CYMV). Inocula contained 0.025 M-phosphate buffer, pH 6.3, 5 µg per ml CYMV and various concentrations of poly-L-ornithine. The protoplasts were stained 44 hours after inoculation	35
3.	Effect of clover yellow mosaic virus (CYMV) concentration in the inoculum on infection of pea mesophyll protoplasts. The inoculum mixtures contained various concentrations of CYMV, 1 µg per ml poly-L-ornithine and 0.025 M-phosphate buffer, pH 6.3. Percentage of the infected protoplasts was determined after 43 hours of incubation.	38
4.	Effect of time of contact between inoculum and protoplasts on infection of pea mesophyll protoplasts with clover yellow mosaic virus. The protoplasts were inoculated as described in the Materials and Methods section except duration of inoculation.	40
5.	Clover yellow mosaic virus multiplication in pea mesophyll protoplasts as determined by infectivity assays on <u>Chenopodium amaranticolor</u> leaves. The protoplasts were inoculated and incubated as described in the Materials and Methods section.	43

LIST OF PHOTOGRAPHIC PLATES

DESCRIPTION

<u>Plate</u>		<u>Page</u>
1.	A light micrograph of protoplasts isolated from pea leaf mesophyll suspended in 0.7 M-mannitol. The scale represents 20 μ m.	54
2.	A. A scanning electron micrograph of pea mesophyll protoplasts. The scale represents 10 μ m	56
	B and C. Fluorescence micrographs of pea leaf mesophyll protoplasts stained with fluorescent antibody to clover yellow mosaic virus (CYMV): B. Inoculated with CYMV. C. Uninoculated. The scales represent 10 μ m.	56

LIST OF ABBREVIATIONS

The following abbreviations for virus names were used:

AMV, alfalfa mosaic virus

BMV, brome mosaic virus

CCMV, cowpea chlorotic mottle virus

CaMV, cauliflower mosaic virus

CGMMV, cucumber green mottle mosaic virus

CMV, cucumber mosaic virus

CPMV, cowpea mosaic virus

CYMV, clover yellow mosaic virus

PEMV, pea enation mosaic virus

PVX, potato virus X

TMV, tobacco mosaic virus

TNDV, tobacco necrotic dwarf virus

TRV, tobacco rattle virus

TRosV, tobacco rosette virus

TYMV, turnip yellow mosaic virus

WCMV, white clover mosaic virus

CHAPTER 1

INTRODUCTION

Conventional studies on various aspects in fundamental and applied plant virology have been conducted using a whole plant or plant tissue. However, the use of intact plant tissue poses several problems in investigating the process of virus infection and multiplication in individual plant cells. The main disadvantages with the plant tissue are: (1) only a small number of cells initially infected by virus, and (2) the cell-to-cell movement of virus in the plant tissue, resulting in asynchrony of infection and multiplication of the virus in different cells. Therefore, to overcome the above disadvantages, and to establish synchronous infection and multiplication of the virus, a large number of individual cells must be initially obtained and infected.

Isolated plant protoplasts (Cocking, 1960; Takebe et al., 1968) are individual naked (devoid of cell wall) cells, and satisfy both the required conditions; a large number of protoplasts in suspension were infected after a brief exposure to virus suspension, and supported synchronous virus multiplication (Takebe and Otsuki, 1969; Takebe, 1975b).

The protoplast system has vast potential for investigating many unsolved problems in plant virology. The establishment of one-step virus growth in this system makes it possible to analyse the consecutive stages of virus multiplication, and to understand the mechanism of interaction between viruses and their strains. It may also prove useful in elucidating the mechanisms of pathological host

responses such as hypersensitivity. This system can also be exploited for breeding virus-resistant plants by regenerating selected plantlets from virus-resistant protoplasts. All these studies, however, demand a high proportion of infected protoplasts, an important prerequisite step for using this system. Therefore, it is extremely important to define the optimal conditions for a maximum infection of protoplasts with a particular virus.

In this study, CYMV was chosen because it is widespread in western North America (Pratt, 1961; Agrawal, 1962), and is an economically important virus. It reduces winter hardiness and yield in clovers (Pratt, 1967). A natural infection of vetch up to 90 percent in the Rocky Mountain House area has been reported (Rao, Hiruki and Matsumoto, 1980). Recent survey data (Hiruki, Rao and Chen, 1979) suggest that the virus is widespread in forage legume crops in Alberta. The virus is extremely stable in vitro, easily sap-transmissible, and reaches a high concentration in the infected host plant.

As a host species pea (Pisum sativum) was chosen, because among leguminous crops, pea in particular, is highly susceptible to the virus. All introduction lines released by the United States Department of Agriculture were susceptible, and developed severe symptoms after CYMV infection (Ford and Bagget, 1965).

The main objectives of this study are: (1) to study the conditions necessary for the large scale isolation of pea leaf mesophyll protoplasts in intact and viable state, (2) to infect the protoplasts with CYMV and demonstrate the virus multiplication in

them, and (3) to examine the effects of certain factors on virus infection of the protoplasts.

CHAPTER 2

LITERATURE REVIEW

Enzymatic isolation of plant protoplasts and their subsequent culture can be considered as one of the most important developments in plant biology in this century. This elegant technique has attracted the attention of plant biologists, especially plant breeders, who are interested in improving crop plants by protoplast fusion; and plant virologists who want to use them for virus infection studies.

In this chapter, current knowledge regarding their isolation and infection with viruses will be reviewed.

2.1 Isolation of Protoplasts

In a plasmolysed cell, plasmalemma retracts from the cell wall and protoplasts as cellular entity become round in the central space of the cell. This is the basis for mechanical isolation of protoplasts. On cutting the tissue containing plasmolysed cells, sometimes it is possible to disrupt the end walls through which intact protoplasts are released. This technique was first applied to isolate water-aloe protoplasts (Klercker, 1892) and later to onion (Plowe, 1931) and beet (Whatley, 1956). Although mechanically isolated protoplasts were used for many important studies of plasmalemma (Vasil, 1976), this method did not become very popular due to its limitations: small number of released protoplasts and restricted use of the method which can only be applied to mature

plant cells but not to meristematic cells.

Cocking (1960), in his first attempt to isolate protoplasts enzymatically, used a crude cellulase from the fungus Myrothecium verrucaria to isolate tomato root protoplasts. This was followed by the isolation of protoplasts in a hypertonic medium (Gregory and Cocking, 1963; 1965; Ruesink and Thimann, 1965; 1966). A real breakthrough in the study of plant protoplasts came when Takebe et al. (1968) developed a method to isolate a large number of tobacco mesophyll protoplasts. Leaf cells were obtained by incubating the epidermis-removed leaves with a crude polygalacturonase from Rhizopus sp. and by digesting the cell walls of isolated mesophyll cells with Cellulase Onozuka P 1500, a crude cellulase preparation from Trichoderma viride. This 'two-step method,' as it is known now, was later used to isolate protoplasts from other higher plants (Otsuki and Takebe, 1969).

The procedure developed by Takebe et al. (1968) was modified later by other workers. In one of the modifications, pectinase and cellulase were used together to obtain tobacco leaf mesophyll protoplasts (Power and Cocking, 1968; 1970; Kassanis and White, 1974). In this 'one-step method,' the separation of cells and digestion of cell walls is achieved simultaneously. The one-step method is simpler than the two-step method, but unlike the latter where protoplasts are obtained only from pallisade tissue, the former prepares protoplasts from both spongy and pallisade tissues. The selective preparation of pallisade protoplasts achieved by the two-step method reportedly gives rise to a higher infection rate than a mixture of protoplasts from spongy and pallisade cells and does not require

potassium dextran sulfate (Takebe et al., 1968). Furthermore, Takebe (1977) indicates that the one-step method is more drastic to mesophyll cells than the two-step method, and produces many small spherical bodies lacking part of the cellular content.

Leaves of some plant species are so vulnerable that shaking during incubation with Macerozyme damages the cells, thereby reducing the number of cells available for further incubation with cellulase. Watts and King (1973) suggested a very simple modification to overcome this problem. Leaves are incubated with Macerozyme at a slow reaction rate, not enough to release the cells but to soften the leaf tissue. Incubation of softened leaves with cellulase released a large number of protoplasts. In this method, like the two-step method, the protoplasts are obtained from both spongy and palisade cells.

Removal of the epidermis increased penetration of the enzyme into the remaining tissues, but some workers found it rather tedious and time consuming. Alternatively, penetration of the enzyme into tobacco mesophyll tissue was facilitated by cutting the tissue into narrow strips (Zaitlin, 1959; Jensen et al., 1971; Vasil, 1976; Chin and Scott, 1979), or by infiltration of the enzyme in vacuum (Nagata and Ishii, 1979). In another effort by Schilde-Rentschler (1972) using an enzyme to digest the cutin of the epidermis, the protoplasts yield thus obtained was significantly reduced. To remove the epidermis, Beier and Bruening (1975) brushed on the lower side of cowpea leaf with Carborundum (320 mesh) and Shepard (1975) stroked the lower side of tobacco leaf with a nylon brush until it was shiny green. There is a considerable risk to damage the cells

by these operations. Evaluating all the procedures, peeling off the epidermis with a pair of forceps appears to be still the best method, although it has its own limitations.

Prolonged incubation of leaves with enzyme should be avoided in protoplast isolation. Treating tobacco leaf tissue with a new enzyme Pectolyase Y23 and Cellulase Onozuka R10, Nagata and Ishii (1979) considerably reduced the incubation time from two hours to 25 minutes.

Along with protecting the integrity of the protoplasts, osmoticum also provides the necessary plasmolysis in the initial stages of protoplast isolation. Two kinds of osmotica; ionic, such as sucrose (Power and Cocking, 1968; Shepard and Totten, 1975) and sorbitol (Watts and King, 1973); and non-ionic, such as mannitol (Takebe et al., 1968) have been used. Meyer (1974) proposed an osmoticum consisting of salts but almost all workers prefer sugars, especially mannitol.

Physiological state of the leaves is one of the determining factors for high yields of stable protoplasts. There have not been many systematic studies to investigate the defined conditions under which different plant species should be grown for protoplasts isolation. However, efforts were made in attempts to define environmental conditions to culture tobacco, tomato and barley plants that serve as reliable sources of protoplasts (Kubo et al., 1975a; Cassells and Barlass, 1978a; Hughes et al., 1978). Plant age also influences the stability of isolated protoplasts and their rate of infection with virus. High percentages of infection were obtained when tobacco protoplasts from younger leaves were inoculated with CCMV than when

protoplasts were obtained from older ones (Motoyoshi et al., 1974b). Watts et al. (1974) suggested some guidelines in selecting leaves of a correct age for obtaining satisfactory protoplasts.

Due to seasonal variations and other cultural practices, each laboratory has to work out its own optimum conditions for growing plants suitable for isolation of a large number of stable and highly susceptible protoplasts. It was suggested that mature leaves should be taken soon after their full expansion from a plant that is in a rapid and uninterrupted growth stage (Watts et al., 1974; Takebe 1977).

The enzymes for protoplast isolation are commercially available. Reagents, equipment and detailed procedures are well reviewed by Sarkar (1977).

2.2 Inoculation and Incubation of Protoplasts

In the 1960's it was thought that protoplasts may offer a potential tool for virus research. Two bases for this perception were the wall-less nature of the protoplasts, and an assumed possibility that they may pick up virus from the surrounding medium. Cocking (1966) demonstrated the ability of tomato fruit protoplasts to pick up TMV, and then two groups simultaneously reported the evidence of TMV infection and its multiplication in tomato fruit protoplasts (Cocking and Ponjar, 1969), and tobacco mesophyll protoplasts (Takebe and Otsuki, 1969).

A reason for Takebe and Otsuki (1969) achieving a rate of infection of protoplasts higher than that of Cocking and Ponjar (1969) was the incubation of TMV with poly-L-ornithine (PLO) prior to

inoculation. The 'indirect method' of inoculation developed by Takebe and Otsuki (1969) has been used extensively by many other workers in inoculating plant mesophyll protoplasts with viruses such as TMV (Coutts et al., 1972; Kassanis and White, 1974), CCMV (Motoyoshi et al., 1973a), CGMMV (Sugimura and Ushiyama, 1975), PVX (Otsuki et al., 1974), TNDV (Kubo and Takanami, 1979), CPMV (Hibi et al., 1975) and CMV (Koike et al., 1977).

Motoyoshi et al. (1974b) reported another method of inoculation known as a 'direct method.' Successful infections of tobacco protoplasts with CPMV (Huber et al., 1977) and CCMV (Sakai et al., 1977); of tomato protoplasts with TMV (Motoyoshi and Oshima, 1975); and of turnip leaf protoplasts with CaMV (Howell and Hull, 1978) were achieved by this method. Motoyoshi et al., (1974b) claim that their method is simple and more efficient than the 'indirect method.' While the 'direct method' is simpler than the 'indirect method,' there is no difference in the infection rates (Kubo et al., 1975b; Rao and Hiruki, 1978; Morris-Krsinich et al., 1979).

Okuno and Furusawa (1978b) applied osmotic shock to barley protoplasts for inoculation with BMV. The authors suggested that increased osmotic pressure caused a shrinkage of protoplasts, thus presumably increasing adsorption and uptake of virus particles by an endocytosis process. Alblas and Bol (1978), applying the same technique, achieved enhanced infection of cowpea protoplasts with AMV; however, there was no effect on infection of turnip leaf protoplasts with TRSV (Morris-Krsinich et al., 1979).

After inoculation with virus, protoplasts are incubated in an incubation medium. Most commonly used incubation medium is of Takebe et al. (1968) which was later modified by Aoki and Takebe (1969).

The medium contains non-organic salts, plant growth regulator and antibiotics, but there is no metabolizable carbon source. Protoplasts suspended in this simple medium support virus multiplication. Some other studies also supported this view: virus multiplied to a considerable extent in protoplasts incubated in mannitol alone (Kassanis et al., 1975); there was no effect on virus yield when the concentrations of nitrogen, phosphorous and potash in the medium were increased to five times the usual concentrations (Kassanis and White, 1974); addition of sucrose to the medium did not increase virus yield (Motoyoshi et al., 1974b); and there was no difference between the photosynthetic activity of protoplasts and that of intact leaf tissue (Nishimura and Akazawa, 1975). These observations suggest that while nutrients in a supporting medium are very important for the stability of protoplasts, they do not contribute towards virus multiplication.

Protoplasts are incubated usually in the continuous light for virus multiplication. The fact that the multiplication of virus in protoplasts is very much inhibited in the dark (Kassanis and White, 1974; Renaudin et al., 1975; Okuno and Furusawa, 1977) suggests that it depends upon the energy supplied by the photosynthesis.

To check the growth of bacteria and fungi during incubation, antibiotics can be used but they are not a substitute for good aseptic techniques. Watts and King (1973) suggested a combination of antibacterial agent such as carbenicillin or gentamicin with antifungal agent nystatin for effective control of these microorganisms. Careful selection of antibiotics and its concentration is very

important, because antibiotics including gentamicin at certain concentrations are inhibitive to both bacterial growth and virus multiplication (Kassanis et al., 1975).

2.3 Factors Affecting Infection of Protoplasts

Many factors in addition to the growth conditions of a source plant, influence the frequency of infection of protoplasts with virus, in particular the conditions of inoculation medium.

The pH of the inoculation medium and the kind of buffer used for inoculation are two important factors in infection of protoplasts. Plant protoplasts carry negative charges (Grout and Coutts, 1974; Nagata and Melchers, 1978); therefore, virus inoculation is done generally in a range of acidic pHs. With most viruses, for example, protoplasts have been inoculated at about pH 5 (Takebe, 1977) and below this value protoplasts are very unstable (Hibi et al., 1975; Motoyoshi and Oshima, 1975; Takebe, 1975a). PVX infection of tobacco protoplasts occur in a broad optimum range around pH 5.8 (Otsuki et al., 1974), and the maximum infection of cowpea protoplasts with CYMV occurred at pH 6.0 (Rao and Hiruki, 1978).

The kind of buffer used for inoculation influences the frequency of infection (Kubo et al., 1974; Motoyoshi and Oshima, 1975; 1976; Mayo, 1978; Morris-Krsinich et al., 1979; Mayo and Roberts, 1979). A benefit of using phosphate buffer (Kubo et al., 1974; Motoyoshi and Oshima, 1975) and tris-HCL buffer (Motoyoshi and Oshima, 1976; Mayo, 1978; Morris-Krsinich et al., 1979) is that these buffers increased optimum pH value of inoculation medium. In the presence of

phosphate buffer, increases in the number and decreases in the size of aggregates containing TRV and attached to tobacco protoplasts were noted in electron microscopy (Kubo et al., 1976). This was interpreted to mean increases in the sites of infection. However, the interaction of the phosphate ions and the plasmamembrane after attachment, may also have its role in the phenomenon (Kubo et al., 1974; 1976).

The addition of PLO in the inoculum is another important factor in obtaining high percentage of virus infection (Takebe and Otsuki, 1969). It is essential or at least stimulatory for infection in almost all virus-protoplast combinations so far tested (Takebe, 1977; Alblas and Bol, 1977; Barker and Harrison, 1977; Rao and Hiruki, 1978; Howell and Hull, 1978; Morris-Krsinich et al., 1979; Kubo and Takanami, 1979). Cassells and Barlass (1978b) using polyethylene glycol (PEG, MW, 6,000) instead of PLO, reported a 60 percent infection of tomato mesophyll protoplasts with TMV and suggested that PEG may induce plasmamembrane fusion, similar to the membrane fusion it induces in protoplasts (Kao and Michayluk, 1974). However, they did not compare the rates of infection influenced by PEG and PLO. All the viruses so far tested except a few viruses including PEMV and BMV, which can infect protoplasts in absence of PLO (Motoyoshi and Hull, 1974; Okuno et al., 1977), have acidic isoelectric points and are negatively charged at the pHs used for inoculation. PLO effects the adsorption of virus particles to protoplasts by neutralizing or even reversing the negative surface charge of the virus particles (Takebe et al., 1975). To achieve the above mentioned change in the charge of the virus particles, it is important that

the virus and PLO be incubated together for at least 10 to 20 minutes before inoculation (Motoyoshi et al., 1974b). In fact, some turbidity developed in the inoculum as a result of the formation of aggregates between PLO, buffer anion and virus particles (Kubo et al., 1976; Mayo and Roberts, 1978; Morris-Krsinich et al., 1979).

Recent investigations suggest that the situation regarding PLO requirement may not be as clear-cut as it is stated above. It is not only the charge on the virus particles that effects infection but also the amount of negative charge on the protoplasts. For example, CPMV and TMV do not need PLO for infection of cowpea protoplasts (Hibi et al., 1975), but the same viruses require PLO for infection of tobacco protoplasts (Huber et al., 1977; Takebe and Otsuki, 1969) suggesting that cowpea protoplasts have relatively lower surface potential than tobacco protoplasts. This suggestion was later confirmed by Nagata and Melchers (1978).

PLO is generally used at a concentration of 1 μg per ml. However, as low as 0.4 μg per ml (Rao and Hiruki, 1978) and as high as 10 μg per ml (Shalla and Peterson, 1973) have also been used. It is generally considered that a PLO concentration higher than 1 μg per ml is deleterious to the protoplasts. The ability of some other polycations to stimulate infection of protoplasts was tested also (Motoyoshi et al., 1974b; Takebe et al., 1975). Only poly-L-lysine was as effective as PLO. However, PLO is most commonly used for this purpose. Molecular weight of PLO is an important factor in determining its effectiveness to stimulate the infection of protoplasts. PLO of molecular weight of 130,000 or higher was more effective than the one with low molecular weight of 90,000 (Motoyoshi et al., 1974b;

Takebe et al., 1975).

PLO-enhanced virus infection of protoplasts appears to be caused by its involvement in two steps of infection processes. The first step, as mentioned earlier, is its effect on the charge on the virus particles, thereby affecting their adsorption to the plasmamembrane. The second step involves the effects of PLO on plasmamembrane causing the uptake of virus particles into the cytoplasm. There are two schools of thought to explain the role of PLO in the second step. The first view is that virus particles are taken up into the protoplasts by pinocytotic activity (Cocking, 1966; 1970; Cocking and Ponjar, 1969; Takebe and Otsuki, 1969; Hibi and Yora, 1972; Honda et al., 1974), and PLO enhances this activity (Otsuki et al., 1972). The second view is that the virus is taken up by a non-physiological process through the PLO-induced lesions of plasmamembrane which serve as sites for virus binding (Burgess et al., 1973a; 1973b).

In support of the plasmamembrane-lesion hypothesis, Motoyoshi et al. (1974b) reported that DEAE dextran, a promotor of pinocytosis, did not increase infection of tobacco protoplasts with CCMV. PEMV infects tobacco protoplasts in the absence of PLO by entering into the protoplasts through the damaged plasmamembrane that had occurred during the preparation of protoplasts (Motoyoshi and Hull, 1974). Kubo et al. (1976) observed no pinocytosis during infection of tobacco protoplasts with TRV and suggested that floccules of the virus and PLO cause breaks in the plasmamembrane. This view was also supported by Kassanis et al. (1977). However, Suzuki et al. (1977), on the other hand, observed endocytosis of polystyrene sphere by tobacco protoplasts and found no evidence of uptake of the spheres

through the lesions on the plasmamembrane.

The virus concentration in the inoculum also influences the efficiency of infection. Within a certain range, an increase in the infection efficiency is proportional to the increase of the virus concentration in the inoculum as found for TMV (Takebe and Otsuki, 1969), PVX (Otsuki et al., 1974), TYMV (Renaudin et al., 1975), and TNDV (Kubo and Takanami, 1979). If increased beyond this range, it does not result in an increased infection rate (Motoyoshi and Oshima, 1975; Morris-Krsinich et al., 1979). Further increase of virus concentration decreases the number of infected protoplasts (Takebe, 1975; Takebe et al., 1975; Morris-Krsinich, 1979), and a possible reason for this decrease could be a limit of another important compound, PLO. Multicomponent viruses, such as PEMV (Motoyoshi and Hull, 1974), BMV-V5 (Motoyoshi et al., 1974a), and BMV wild type (Okuno et al., 1977), require a high virus concentration for a maximum infection. The concentration of virus in the inoculum represents the number of virus particles available for each protoplast. For infection of one tobacco protoplast, 10^5 TMV particles (Takebe and Otsuki, 1969) and $3-5 \times 10^5$ CCMV particles (Motoyoshi et al., 1973b) are needed in the inoculum, and cowpea mesophyll protoplast requires 1.3×10^5 CPMV particles (Hibi et al., 1975). However, only those particles adsorbed to the protoplast are important for infection and the number of these particles is much less than the number of the particles available to each protoplast (Takebe and Otsuki, 1969; Motoyoshi et al., 1973b; Zhuravlev et al., 1975; Hibi et al., 1975). Even after adsorption of virus particles, only small amounts of the particles adsorbed to

individual protoplasts undergo further dissociation, suggesting that a small number of virus particles are actually involved in infection (Wyatt and Shaw, 1975).

In fact, the effects of different factors affecting the efficiency of virus infection is very much interrelated. Presence or absence, and concentration of a component in the inoculum, determine the behaviour of the other component(s). Electrostatic force plays a very important role in the process of virus adsorption by the protoplasts (Motoyoshi et al., 1974a; Takebe et al., 1975). Negatively charged viruses form a complex with PLO, and net charge of this complex depends upon the ratio of the concentrations of virus and PLO which should be positive if this complex is to adsorb to the protoplasts (Motoyoshi et al., 1974a). Therefore, it is not simply the concentration of PLO or virus but a ratio of their concentrations that is important for a maximum infection. A decrease in the infection rate due to an increase in the virus concentration in the inoculum, beyond optimum concentration, may also be explained as a result of an increase in the net negative charge of a virus-PLO complex. Recent studies have indicated that the pH of the inoculation medium influences the required concentrations of PLO and BMV. Infection of the protoplasts with the virus at low pH is independent of PLO but requires a high concentration of the virus (Motoyoshi et al., 1974a; Okuno et al., 1977). However, at high pH it is PLO-dependent and requires only a low concentration of the virus (Okuno and Furusawa, 1978a). In the presence of phosphate ions, TRV infection of tobacco protoplasts is inversely related to the concentration of protoplasts, but not when citrate is used (Mayo, 1978).

2.4 Detection of Virus Infection and Multiplication in Protoplasts

A protoplast can be considered as infected if it contains the progeny virus particles. Three most commonly used methods for determination of infection and multiplication of virus in protoplasts include infectivity assay (Aoki and Takebe, 1969), electron microscopy (Cocking and Ponjar, 1969), and fluorescent antibody staining (Otsuki and Takebe, 1969).

Some other methods have been used for certain reasons or simply for convenience. Sucrose density gradient analysis was applied to determine the amount of virus multiplied in protoplasts (Motoyoshi et al., 1974a; Motoyoshi and Hull, 1974; Renaudin et al., 1975; Morris-Krsinich et al., 1979). The virus concentration in the protoplasts has also been assayed by the serological methods (Coutts et al., 1972; Kassanis and White, 1974; Kubo et al., 1975b) and by the radioisotope technique (Motoyoshi and Hull, 1974; Howell and Hull, 1978). The infection of Brassica protoplasts with TYMV caused chloroplasts to aggregate and form a polyplast, which was exploited as a characteristic feature of infection in determining the efficiency of infection by light microscopy (Renaudin et al., 1975).

Infectivity assay is one of the most favoured methods for determining virus multiplication in the protoplasts. A typical growth curve of TMV in the protoplasts inoculated in vitro is as follows (Takebe and Otsuki, 1969; Takebe, 1975; 1977): Small infectivity obtained at zero hour represents the virus adsorbed to protoplasts. This is followed by a decrease in the infectivity representing uncoating of the virus particles. The production of progeny virus particles becomes evident six hours after inoculation and virus multiplies

exponentially until 12 hours post infection. After this time the rate of virus multiplication slows down and reaches a plateau. By this method, similar results were obtained also for CPMV in cowpea protoplasts (Hibi et al., 1975; Beier et al., 1977), BMV in barley protoplasts (Okuno et al., 1977), and CYMV in cowpea protoplasts (Rao and Hiruki, 1978). In many virus-protoplast combinations, a virus growth curve may deviate from the typical described for TMV in tobacco protoplasts. No infectivity at zero hour after inoculation was found in tobacco protoplasts inoculated with CMV (Otsuki and Takebe, 1973) and CCMV (Motoyoshi et al., 1973a); and there was no initial drop of infectivity of PVX in tobacco protoplasts (Otsuki et al., 1974). In both cases, limited sensitivity of the host was cited as a probable reason. In some virus-protoplast combinations, viruses show a longer lag period in the protoplasts, as reported in the following: TYMV in Brassica protoplasts (Renaudin et al., 1975), CGMMV and CPMV in tobacco protoplasts (Sugimura and Ushiyama, 1975; Huber et al., 1977), CaMV and TRSV in turnip leaf protoplasts (Howell and Hull, 1978; Morris-Krsinich et al., 1979). A longer lag period of CPMV may be due to a limited number of the virus particles entering the protoplasts, or an extended period required for uncoating of infective particles (Huber et al., 1977), or in the case of CaMV slow virus replication (Howell and Hull, 1978).

Electron microscopy, a very useful means of studying virus infection of protoplasts, is used for four types of observations made on thin sections: (1) to study the process of virus uptake by protoplasts; (2) to study the time course of multiplication of viruses; (3) to show the presence of progeny virus particles in the infected

protoplasts; and (4) to observe, if there is any, structural modification of cell organelle(s) due to infection. Regarding the process of virus uptake by protoplasts, electron microscopic observations demonstrated very clearly that the virus particles adsorb to plasma-membrane and are then taken up by a pinocytotic process (Hibi and Yora, 1972; Otsuki et al., 1972; Honda et al., 1974; 1975). However, an interpretation against this theory was presented in favour of lesions of plasmamembrane as the sites for virus binding (Burgess et al., 1973a; 1973b). The results of the electron microscopic studies of the time course of multiplication of TMV (Otsuki et al., 1972), CMV (Honda et al., 1974), and PVX (Honda et al., 1975) are in agreement with the results obtained by infectivity assay or by fluorescent antibody staining (Takebe and Otsuki, 1969; Otsuki and Takebe, 1973; Otsuki et al., 1974). To prove virus multiplication, a section from an infected protoplast must contain a large number of virus particles (Burgess et al., 1974). Infections of tobacco protoplasts with TRV (Harrison et al., 1976), cowpea mesophyll protoplasts with CMV (Koike et al., 1977), and tobacco protoplasts with TNDV (Kubo and Takanami, 1979) meet this requirement. Electron microscopy is used also to examine the ultrastructural changes in protoplasts following infection with viruses, and to compare these changes with cellular ultra-structural changes of leaf tissue infected with the same viruses. The laminate inclusion body developed in the cells of leaf tissue infected with PVX (Shalla and Shepard, 1972) is seen in thin sections of the tobacco protoplasts infected with the virus (Shalla and Peterson, 1973; Otsuki et al., 1974). A cytopathological structure consisting of vesicles surrounded by electron-dense material, similar to that found

in cowpea leaves infected with CPMV (De Zoeten et al., 1974) has been shown in CPMV-infected cowpea and tobacco protoplasts (Hibi et al., 1975; Huber et al., 1977). Sugimura and Ushiyama (1975) observed vesicles in the mitochondria of tobacco protoplasts infected with CGMMV; these vesicles were similar to those observed by Hatta et al. (1971) in the cells of some other host plants infected with the virus. However, some ultrastructural changes characteristic of virus infection may not necessarily develop in protoplasts. For example, chloroplast degeneration, a conspicuous feature of CMV infection of leaf tissue (Misawa and Ehara, 1966), did not occur in the CMV-infected tobacco protoplasts at least for 48 hours after inoculation (Honda et al., 1974).

Fluorescent antibody is prepared by conjugating fluorescein isothiocyanate (FITC) to γ -globulin. An advantage of the fluorescent antibody technique lies in its ability to demonstrate the infection of individual protoplast. Protoplasts fixed on a glass slide can be stained with a drop of fluorescent antibody specific to the virus used for inoculation of the protoplasts. Infected protoplasts will fluoresce as yellow green under a fluorescence microscope. In virus multiplication studies, the first fluorescence appears as weak fluorescent specks and later the cytoplasm is filled with numerous bright fluorescent spots (Kubo et al., 1975b), or weak fluorescent specks increasing in size, or the fluorescence is seen throughout the cytoplasm, forming a network due to the background of unstained dark chloroplasts (Koike et al., 1977; Rao and Hiruki, 1978). The fluorescent antibody staining technique is applicable to the determination of the percentages of infected protoplasts as a function of time, and gives rise to the

results similar to those obtained by infectivity assay (Hibi et al., 1975; Huber et al., 1977). Recently, Cassalls and Gatenby (1975) reported an interesting modification of the technique using Lissamine Rhodamine B (RB 200), an antibody label. The RB 200-conjugated antibody produced less autofluorescence than that of FITC-antibody. Both the FITC and RB 200 conjugated antibodies may be used together to demonstrate the infection of individual protoplast with two different viruses, because the spectral properties of these fluorochromes are entirely different, while double infection of tobacco protoplasts with serologically unrelated viruses (Otsuki and Takebe, 1976b) and strains of a virus (Otsuki and Takebe, 1976a; Barker and Harrison, 1978) still can be demonstrated by using only the FITC-conjugated antibody and simple mathematical calculations.

All these methods differ in their sensitivities in detecting the infection of protoplasts and the extent of virus multiplication. Tobacco protoplasts infected with viruses that multiply to a very low extent, such as PEMV (Motoyoshi and Hull, 1974) and CGMV (Sugimura and Ushiyama, 1975), fluoresce when stained with the specific antibody but no infectivity could be detected. In contrast, low-degree-multiplication of CaMV in turnip leaf protoplasts could not be detected by the fluorescent antibody technique and the infectivity assay; therefore, a radioisotope technique had to be applied to assay the virus (Howell and Hull, 1978). The virus yields determined by comparing the infectivity of protoplast extracts with that of the known concentrations of purified virus are generally higher because the purified virus has the lower specific infectivity than that of the protoplast extracts (Kubo et al., 1975b) and the virus yields estimated

by electron microscopy are higher than those of infectivity assay, because both normal and broken particles are taken into account (Coutts et al., 1972). Shalla and Peterson (1973) suggested that electron microscopy is more sensitive than the fluorescent antibody technique that was used for detecting infection of tobacco protoplasts with PVX. However, using the same virus-protoplast system, Honda et al. (1974) did not find such discrepancy. Every method has its own merits and demerits; most workers have used these in combinations as they compliment each other.

CHAPTER 3

MATERIALS AND METHODS

3.1 Isolation of Pea Mesophyll Protoplasts

A. Source plant

Seeds of Alaska pea were sown in a 3:2:1 soil mix (3 parts loam: 2 parts peat: 1 part sand) in 10 cm clay pots in a greenhouse at 23⁰ to 25⁰C. After germination, the plants were transferred to an incubator (Model E30, Percival Co., Boone, Iowa, U.S.A.) and were kept at 23⁰C with a daily light period of 14 hours at approximately 8,000 lux supplied by six fluorescent tubes (F 24712 kw/40, Sylvania Ltd., Montreal, Canada) and two 40 watt incandescent bulbs (Westinghouse Canada Ltd.), and at 21⁰C with a dark period of 10 hours.

B. Isolation of protoplasts

Fully expanded leaves from three-week-old plants were used for protoplast isolations. After surface-sterilization with 70 percent ethanol and two washings with double-distilled water, the lower epidermis of the leaves was removed with a pair of forceps and the tissue was placed with its epidermis-stripped side down in a Petri dish containing 0.05 percent Macerozyme R-10 (Kinki Yakult Manufacturing Co.) and 1 percent Cellulase Onozuka R-10 (Kinki Yakult Manufacturing Co.) in 0.7 M-mannitol, pH adjusted to 5.5 with 2 N KOH. After two hours of incubation in a water bath at 30 C, the content of the Petri dish was gently swirled and the incubation was

continued for another 30 minutes. The mixture was filtered through a double layer cheesecloth and the protoplasts were collected by centrifuging the filtrate for two minutes at 700 rpm. The protoplasts were washed twice with 0.7 M-mannitol and finally resuspended in 0.7 M-mannitol. The percentage of intact protoplasts was determined by counting about 300 protoplasts, and the yield of protoplasts per gram of fresh leaves was calculated by using a haemocytometer.

Protoplasts were also isolated by the method of Nagata and Ishii (1979). Lower epidermis of the leaves was removed instead of cutting them into narrow slices. The tissues were then placed with their epidermis-removed side down in a Petri dish containing an enzyme solution. The enzyme solution contained Pectolyase Y23 (Kikkoman Shoyu Co. Ltd., Noda, Chibaken, Japan) and 2 percent Cellulase R-10 in 0.7 M-mannitol, pH 5.5. After 35 to 45 minutes of incubation at 30°C, with occasional gentle swirlings, most of the cells released protoplasts which were then collected and washed as described earlier.

In some experiments protoplasts were also isolated by the method of Watts and King (1973).

3.2 Virus

The vetch strain of CYMV (Hiruki et al., 1976) was purified from infected Alaska pea leaves as follow: Frozen leaves were homogenized in a Waring Blender with 0.1 M-phosphate buffer, pH 7.0, containing 0.5 percent ascorbic acid. The homogenate obtained was passed through a double layer of cheesecloth, and sap was clarified using chloroform and butanol in the ratio of 1:1. Clarified

sap was centrifuged at 7,700 g for 10 minutes, and the virus was precipitated from the supernatant liquid by incubating it with 3 percent polyethylene glycol (PEG, mol. wt. 6,000) and 0.6 percent NaCl for two hours at 4°C. The virus was pelleted by centrifuging at 5,100 g for 10 minutes. The pellet was suspended in 0.025 M-phosphate buffer, pH 7.0. The suspension was centrifuged at 12,000 g for 10 minutes and the supernatant liquid was subjected to a high-speed centrifugation at 78,000 g for 90 minutes. The pellet after high-speed centrifugation was suspended in 0.025 M-phosphate buffer, pH 7.0 and the suspension was centrifuged at 12,000 g for 10 minutes. The supernatant liquid containing the virus was obtained and stored at 4°C. A Sorvall RC2-B refrigerated centrifuge was used for low-speed centrifugation and a Beckman ultracentrifuge (L5-75) for high-speed centrifugation.

3.3 Inoculation of Protoplasts

Fresh preparations of CYMV stored at 4°C for not longer than two weeks were used for inoculation of the protoplasts. Unless otherwise stated, the virus was diluted to a concentration of 10 µg per ml in 0.05 M-phosphate buffer, pH 6.3, containing 0.7 M-mannitol, and then PLO (mol. wt. 150,000, Pilot Chemical Co., Boston, Mass., U.S.A.) was added to a concentration of 2 µg per ml. This mixture was incubated for 20 minutes at 25°C and then added to an equal volume of a protoplast suspension. Thus, the final concentrations of virus, PLO and protoplasts were 5 µg per ml, 1 µg per ml, and approximately 2.5×10^5 per ml, respectively.

For the direct method of inoculation, the virus was diluted to a concentration of 5 μg per ml in 0.025 M-phosphate buffer, pH 6.3, containing 0.7 M-mannitol, and then PLO was added to a concentration of 1 μg per ml. The inoculation mixture was incubated at 25°C for 20 minutes prior to suspending freshly pelleted protoplasts. The final concentrations of the virus, PLO and protoplasts were the same as those in the indirect method. Except an increased mannitol concentration of 0.85 M, all other conditions were same as in the indirect method for inoculation by the osmotic shock method (Okuno and Furusawa, 1978b).

In all the methods used for inoculation, the protoplasts were incubated with the inoculum for 20 minutes at 25°C with occasional swirlings and then they were separated from the unadsorbed virus particles by three washes in 0.7 M-mannitol containing 10 mM CaCl_2 . Washed protoplasts were resuspended in the incubation medium of Aoki and Takebe (1969), at a concentration of approximately 2.5×10^5 per ml. They were incubated in 10 ml portions in 125 ml Erlenmeyer flasks at 25°C under continuous illumination (approximately 1,000 lux) from fluorescent tubes.

3.4 Infectivity Assay

After the required incubation times, inoculated protoplasts were collected by centrifugation at 100 g for two minutes, washed once with 0.7 M-mannitol, repelleted and frozen at -20°C for infectivity assay. The frozen protoplasts were thawed and homogenized in 0.2 ml of 0.01 M-phosphate buffer, pH 7.0, using glass tissue grinders (Bellco Biological Glassware, Vineland, U.S.A.). Homogenates were

centrifuged at 12,000 g for 10 minutes and the supernatant liquid containing the virus was applied to eight half-leaves of Chenopodium amaranticolor with a glass spatula after dusting with Carborundum (600 mesh). Local lesions were counted six days after inoculation.

3.5 Fluorescent Antibody Preparation

A. Production of immunoglobulin

Antiserum to CYMV was produced by injecting the purified virus to a San Juan rabbit. The rabbit received two intramuscular injections at 10-day-intervals with 3 mg virus each that had been emulsified with Freund's complete adjuvant. Three intravenous injections were then given with 2 to 3 mg virus per 1 to 1.5 ml per injection at weekly intervals. The rabbit was bled 10 days after the last injection. The antiserum obtained had a titer of 1/1024. The protein concentration of the globulin fraction, isolated from the antiserum by ammonium sulfate precipitation (Campbell et al., 1970), was adjusted to 1 percent.

B. Conjugation with fluorescent isothiocyanate (FITC)

Conjugation of the globulin fraction of the antiserum with FITC (ICN Pharmaceuticals Inc., Ohio, U.S.A.) was carried out as described by Otsuki and Takebe (1969). Non-specific staining of the protoplasts was reduced by absorbing the conjugated antibody with acetone-extracted powder of healthy pea leaves. The conjugated antibody had a titer of 1/256, and a FITC/protein molar ratio of 1.9 as determined from the absorbancy values at 495 nm and 280 nm.

3.6 Staining of Protoplasts with Fluorescent Antibody

After the desired time of incubation, protoplasts were washed with 0.7 M-mannitol containing 10 mM CaCl_2 , and a thick drop of protoplasts suspension was placed on a glass slide smeared with Meyer's albumin. The protoplasts were spread on the glass slide by a gentle rotary motion and then quickly dried in a current of warm air. They were then fixed in 95 percent ethanol for 10 minutes and washed with PBS for 15 minutes. After blot drying, a drop of fluorescent antibody (1/8 dilution) was added to cover the protoplasts, and the slide was incubated at 37°C for 90 minutes. After staining, the protoplasts were washed for one hour in continuously stirred PBS, and then mounted with 40 percent glycerol in PBS. A Carl Zeiss Universal microscope equipped with exciter filters BG12 and BG3 and barrier filters 65 and 50 were used to examine the stained protoplasts.

3.7 Scanning Electron Microscopy

Freshly isolated protoplasts were washed with 0.7 M-mannitol and then fixed for three hours with 3 percent glutaraldehyde in 0.025 M-phosphate buffer, pH 7.0, containing 0.6 M-mannitol. They were then washed twice with 0.025 M-phosphate buffer, pH 7.0, and post-fixed in 2 percent osmium tetroxide for two hours. The protoplasts were then washed as before. The fixed protoplasts were passed through a graded series of ethanol, and then through 40 percent and 85 percent amyl acetate. They were then dried by the critical point method (Anderson, 1951) and were examined with a Cambridge Stereoscan S₄ electron microscope operated at 20KV.

CHAPTER 4

RESULTS

4.1 Method of Isolation

Pea mesophyll protoplasts were isolated by both the 'one-step' and the 'modified two-step' methods. All these methods yielded approximately 1 to 1.5×10^7 protoplasts per gram fresh weight of leaves (Table 1) and 78 to 85 percent of the protoplasts isolated were generally intact. Micrographs of isolated protoplasts as observed with light and scanning microscopes are shown in Plates 1 and 2A, respectively. A difference in these isolation methods is in the time required for liberation of the protoplasts from mesophyll cells, which ranged from 35 minutes to three hours. However, their infection rates with CYMV remained approximately the same. For subsequent experiments, therefore, protoplasts were isolated by the 'one-step' method as described in the Materials and Methods section because Pectolyase Y23 was not commercially available at the time of this investigation.

4.2 pH of Inoculum

The effect of pH of inoculation medium on infection of pea protoplasts was determined by inoculating the protoplasts at different pH values (5.7 to 7.5) of 0.025 M-phosphate buffer. An increasing number of the protoplasts were infected as pH was raised from 5.7 to 6.3, which was an optimal range, and the number of infected protoplasts decreased sharply at higher pH values (Figure 1).

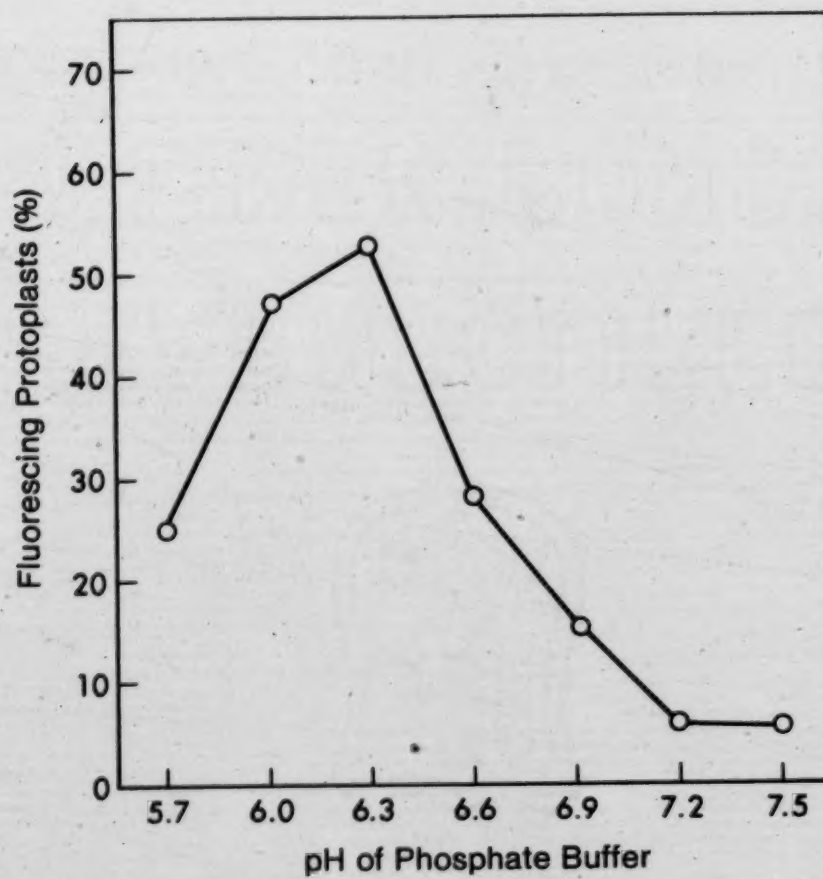
Table 1. Comparison of different methods used for isolation of pea leaf mesophyll protoplasts.

Procedure	Lower epidermis	Macerozyme R10 (%)	Pectolyase Y23 (%)	Cellulase R10 (%)	Yield protoplasts/g*	Time required for liberation of protoplasts
One-step	Peeled	--	0.1	2	$1-1.5 \times 10^7$ **	35 minutes
One-step	Not peeled	--	0.1	2	$1-1.5 \times 10^7$	45 minutes
One-step	Peeled	0.05	--	1	$1-1.5 \times 10^7$	2 hours
Modified two-step	Peeled	1	--	2	$1-1.5 \times 10^7$	3 hours

* Fresh weight of leaves used.

** Protoplasts were counted using a haemocytometer after washing twice with 0.7 M-mannitol.

Figure 1. Effect of pH of inoculation buffer on clover yellow mosaic virus (CYMV) infection of pea mesophyll protoplasts. The protoplasts were inoculated with 5 μ g per ml CYMV in the presence of 1 μ g per ml poly-L-ornithine. The protoplasts were stained with fluorescent antibody specific to CYMV after 44 hours of incubation.



4.3 Kind of Buffer

Kind of buffer also influences the infection of protoplasts (Kubo et al., 1974; Motoyoshi and Oshima, 1976). Two different buffers; 0.01 M-citrate buffer, pH 6.0, used for infection of cowpea protoplasts with CYMV (Rao and Hiruki, 1978), and 0.025 M-phosphate buffer, pH 6.3, were compared to determine their effects on infection. Percentages of CYMV infection from three experiments for the citrate/phosphate buffers were 37/52, 40/57 and 38/53, indicating that the presence of phosphate in the inoculum results in the frequency of infection higher than that of citrate.

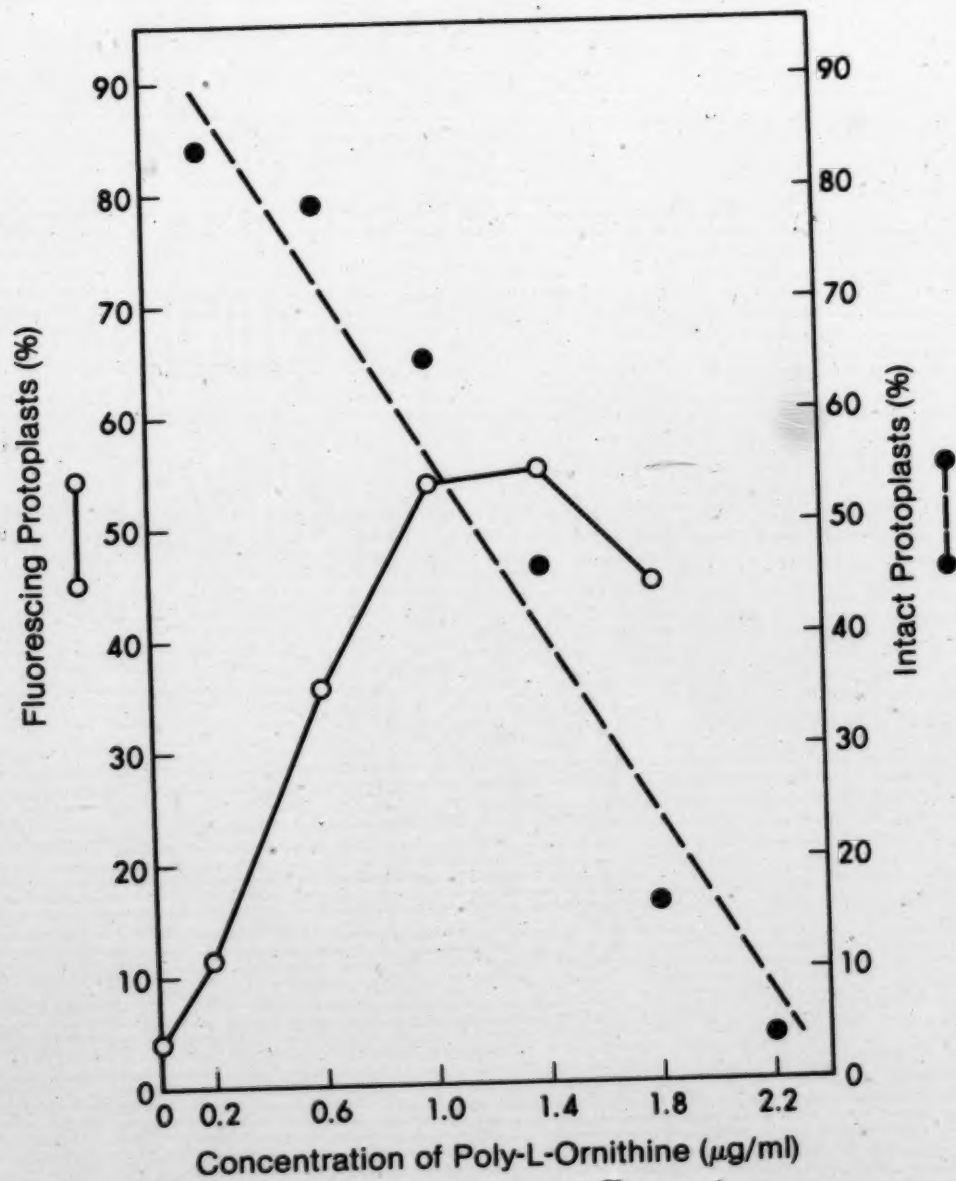
4.4 Concentration of PLO

Very little infection occurred when the protoplasts were inoculated in the absence of PLO (Figure 2). Therefore, experiments were conducted to investigate the effects of PLO concentration on survival and infection of the protoplasts. The optimum concentration of PLO for infection was 1.0 to 1.4 μg per ml. Below and above this range of PLO concentration, the number of infected protoplasts was decreased. As a higher percentage of protoplasts was damaged at 1.4 μg per ml than at 1.0 μg per ml, the protoplasts were inoculated at a PLO concentration of 1.0 μg per ml in the subsequent experiments.

4.5 Methods of Inoculation

Otsuki et al. (1972), using the 'indirect method' reported that sedimentation and resuspension of tobacco protoplasts immediately

Figure 2. Effect of poly-L-ornithine concentrations in the inoculum on survival of pea mesophyll protoplasts and their infection with clover yellow mosaic virus (CYMV). Inocula contained 0.025 M-phosphate buffer, pH 6.3, 5 μ g per ml CYMV and various concentrations of poly-L-ornithine. The protoplasts were stained 44 hours after inoculation.



before inoculation with TMV is essential for a high frequency of infection.

The 'direct method' applied for inoculation of tobacco protoplasts with CCMV (Motoyoshi et al., 1974), and the 'osmotic shock method' applied for inoculation of barley protoplasts with BMV (Okuno and Furusawa, 1978) later resulted in higher frequencies of infection than that of the 'indirect method.' When these three methods were compared for their efficiency of infection in the CYMV-pea protoplast system, however, there was little difference amongst them. In three experiments, the average percentages of infection by the 'indirect method,' the 'direct method' and the 'osmotic shock method' were 44, 43 and 46, respectively. In view of these results, the 'direct method' was used subsequently.

4.6 Concentration of Inoculum

Frequency of infection of the protoplasts was influenced by the concentration of CYMV in the inoculum (Figure 3). The frequency increased with the concentration of the virus in the inoculum up to a concentration of 3 μ g per ml. At this concentration, 56 percent of the protoplasts were infected. Further increase in the virus concentration did not improve the frequency of infection.

4.7 Duration of Inoculation

The time of incubation of protoplasts with inoculum also influenced the percentage of infection. To examine the effects of

Figure 3. Effect of clover yellow mosaic virus (CYMV) concentration in the inoculum on infection of pea mesophyll protoplasts. The inoculum mixtures contained various concentrations of CYMV, 1 μ g per ml poly-L-ornithine and 0.025 M-phosphate buffer, pH 6.3. Percentage of the infected protoplasts was determined after 43 hours of incubation.

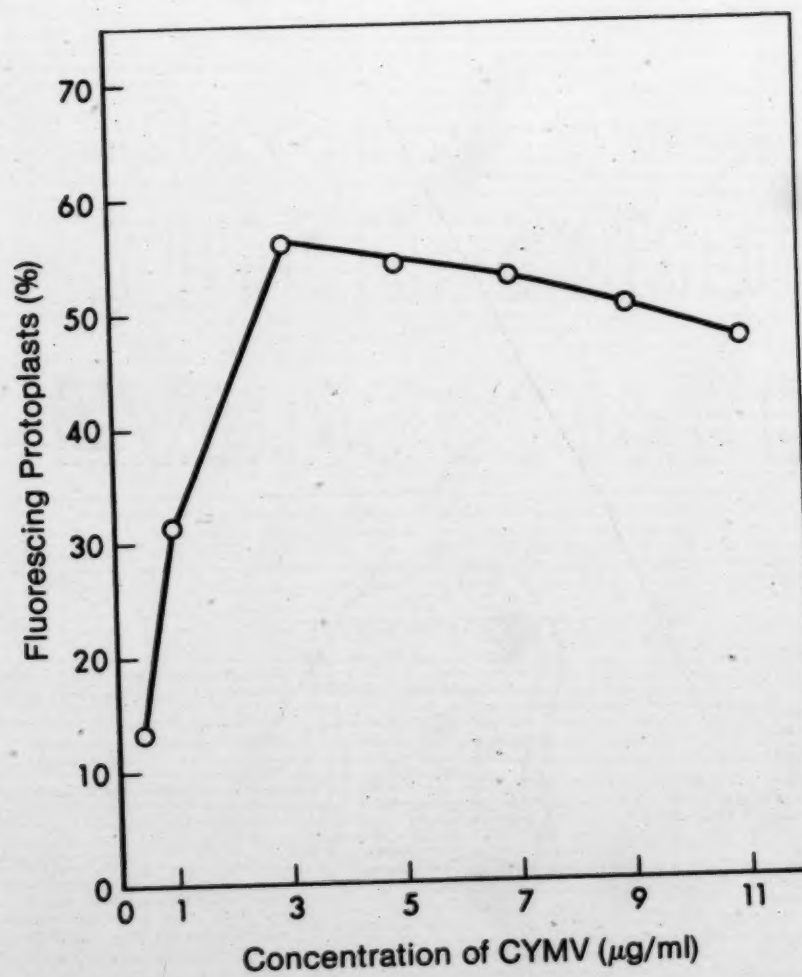
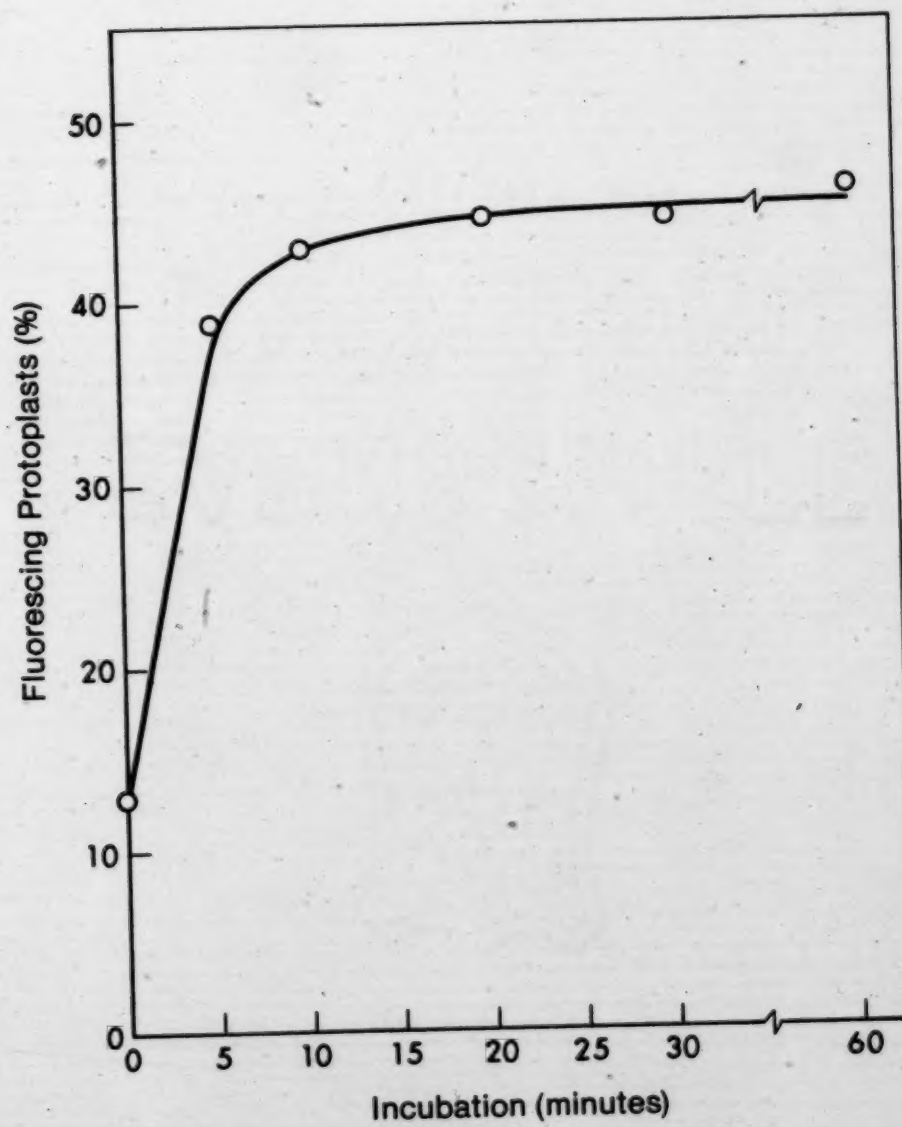


Figure 4. Effect of time of contact between inoculum and protoplasts on infection of pea mesophyll protoplasts with clover yellow mosaic virus. The protoplasts were inoculated as described in the Materials and Methods section except duration of inoculation.



duration of inoculation, the protoplasts were incubated for different periods of time (0 to 60 minutes) with a mixture of CYMV and PLO which had been pre-incubated for 20 minutes. It was found that 10 minutes of incubation gave a relatively good infection rate, and longer incubation than this did not change the rate considerably (Figure 4).

4.8 Multiplication of CYMV

The extent of multiplication of CYMV in the protoplasts was determined by two methods; namely, infectivity assay of protoplast extracts and fluorescent antibody staining of inoculated protoplasts. Figure 5 shows the time course of the virus multiplication as obtained by assaying the infectivity in the protoplasts at the various times after inoculation. A small amount of infectivity detected immediately after inoculation could be attributed to the virus particles simply adsorbed to the protoplasts. This initial infectivity was followed by a decrease in the infectivity six hours after inoculation. It increased very rapidly between 12 and 24 hours and then slowed down. The percentage of infected protoplasts was determined by staining the protoplasts with fluorescent antibody specific to CYMV at intervals after inoculation. The first fluorescence was observed 12 hours after inoculation and 10 percent of the protoplasts were found to be infected (Table 2). During the following 12 hours, the percentage of infected protoplasts increased very rapidly from 10 percent to 38 percent, and then the increase was slowed down.

The first yellow-green fluorescence due to CYMV antigen

Figure 5. Clover yellow mosaic virus multiplication in pea mesophyll protoplasts as determined by infectivity assays on Chenopodium amaranticolor leaves. The protoplasts were inoculated and incubated as described in the Materials and Methods section.

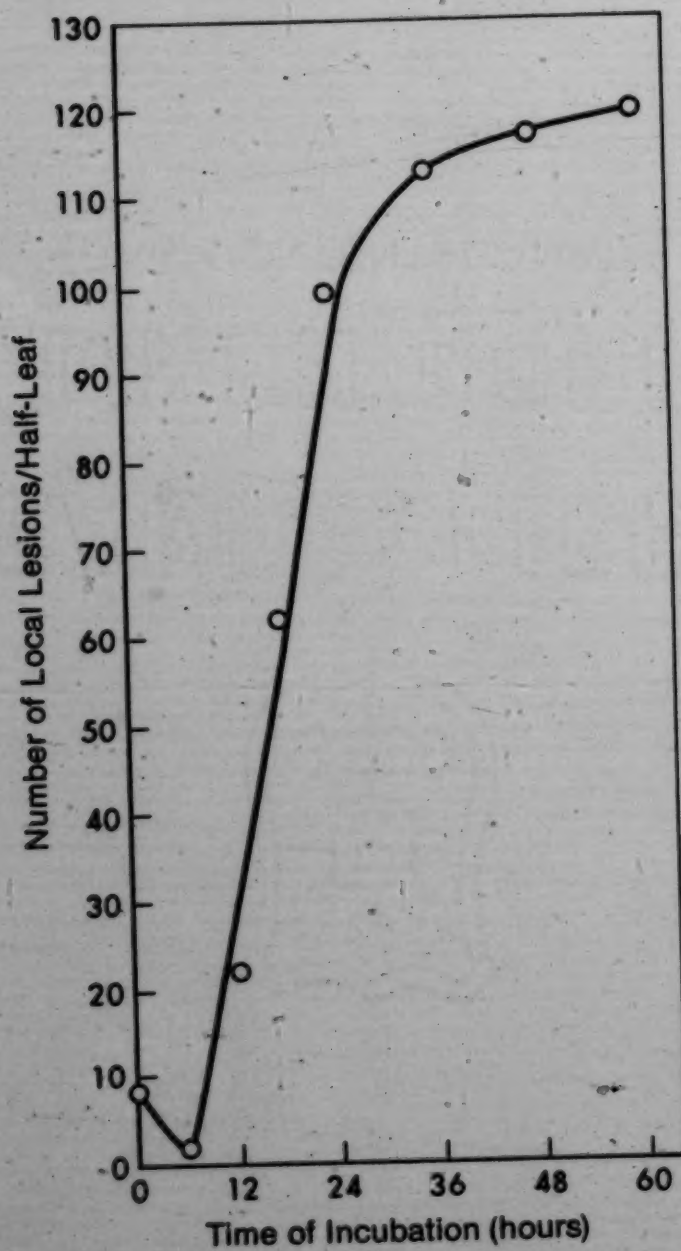


Table 2. The time-course of clover yellow mosaic virus multiplication in pea mesophyll protoplasts as determined by fluorescent antibody technique.

Time after inoculation (hours)*	Fluorescing protoplasts (%)
0	0
12	10
24	38
36	44
60	45

* Protoplasts were inoculated and incubated as described in the Materials and Methods section.

was visible at 12 hours as weak fluorescent specks. After 40 hours of incubation the fluorescence was distributed throughout the cytoplasm, and it appeared as a network due to unstained dark chloroplasts (Plate 2B). There was no such fluorescence when uninoculated protoplasts were treated after 40 hours of incubation with conjugated CYMV antibody (Plate 2C).

CHAPTER 5

DISCUSSION

The present study has shown that pea mesophyll protoplasts can be infected with CYMV in the presence of PLO after brief incubation. When the 'two-step method' (Takebe et al., 1968) was tested in a preliminary test, significant cell damage occurred during shaking of pea tissue with Macerozyme, consequently very few cells were available for the subsequent cellulase treatment. In contrast, the 'modified two-step' (Watts and King, 1973) and 'one-step' (Power and Cocking, 1970) methods required only gentle swirlings, and the damage was minimal. In addition, the 'one-step' method was simple and yielded a large number of protoplasts. All the methods used in this investigation resulted in similar protoplast yields but differed in the liberation time of protoplasts (Table 1). Use of Pectolyase Y23 considerably reduced the time of protoplasts isolation from two hours to 35 minutes. Although the enzyme was used previously to isolate tobacco protoplasts (Nagata and Ishii, 1979), this is the first report of isolation of pea protoplasts after a short exposure to the enzyme.

In this investigation up to 57 percent of pea mesophyll protoplasts were infected with CYMV under the inoculation conditions described in the Materials and Methods section. The observations that virus antigen was detectable 12 hours after inoculation (Table 2), and that extensive fluorescence was observed 40 hours after inoculation (Plate 2B), indicated that the protoplasts inoculated in vitro

supported active virus multiplication. Synchronous nature of infection and virus multiplication was evident from a rapid increase in infectivity between 12 and 24 hours, and from the observation that a majority of the cells fluoresced within 24 hours of inoculation. The result of a time-course study of virus multiplication in the protoplasts as monitored by the infectivity assay and fluorescence microscopy was similar to that of some other virus-protoplast systems (Takebe and Otsuki, 1969; Hibi et al., 1975; Okuno et al., 1977).

In the investigation of the effects of some factors on infection of pea protoplasts with CYMV, PLO was found necessary for infection (Figure 2). Very little infection occurred when it was omitted from the inoculum. PLO was also reportedly essential in many other virus-protoplast combinations (Motoyoshi et al., 1973a; Otsuki and Takebe, 1973; Otsuki et al., 1974; Motoyoshi and Oshima, 1975; Kubo et al., 1975b; Motoyoshi et al., 1975; Barker and Harrison, 1977; Huber et al., 1977; Alblas and Bol, 1977; Rao and Hiruki, 1978; Kubo and Takanami, 1979; Morris-Krsinich et al., 1979). While all these viruses that require PLO for infection were negatively charged at the pH values used for inoculation of the protoplasts, other viruses that were positively charged at their inoculation pHs do not require PLO for infection (Motoyoshi and Hull, 1974; Okuno et al., 1977). Recent investigations, however, indicated that it was not only the charge on virus particles that influences the requirement for PLO, but also the amount of negative charge of the protoplast membrane. Negatively charged CPMV and TMV did not require PLO for infection of cowpea protoplasts (Hibi et al., 1975). However, the same viruses required PLO for infection of tobacco protoplasts

(Huber et al., 1977; Takebe and Otsuki, 1969). The mechanism of the PLO-enhanced infection is not very well understood, although it appears that PLO acts both on virus particles and protoplasts. Upon mixing, it forms a complex with the negatively charged viruses (Kubo et al., 1976; Takebe, 1975b; Rao and Hiruki, 1978), facilitating the virus adsorption to the plasmamembrane. It also enhances the uptake of virus particles through enhanced pinocytotic activity of protoplasts (Cocking, 1966; 1970; Hibi and Yora, 1972; Otsuki et al., 1972), or through plasmamembrane lesions that serve as the sites for virus binding (Burgess et al., 1973a; 1973b).

The kind of buffer used for infection considerably influences the infection (Kubo et al., 1974; Takebe, 1977). Various buffers were used to test the infection efficiency of protoplasts (Kubo et al., 1974; Motoyoshi and Oshima, 1975; Morris-Krsinich et al., 1979). Phosphate buffer increased infection of pea mesophyll protoplasts with CYMV to a higher percentage than did citrate buffer. The mechanism of phosphate-enhanced virus infection of intact tissue (Yarwood, 1952), and that of protoplasts (Kubo et al., 1974) is not completely understood, although some postulations were made. This could be due to an increased number of sites of infection (Kubo et al., 1976), as aggregates containing TRV and attaching to tobacco protoplasts were greater in number and smaller in size when phosphate instead of citrate was included in the inoculum. However, the effects of phosphate on the plasmamembrane after attachment may also be involved in the enhanced infection (Kubo et al., 1974; 1976).

The response of pea protoplasts at different pH values of

the inoculation medium towards infection with CYMV was considerably different from most other virus-protoplast combinations. The optimal pH value of 6.3 for infection of pea protoplasts was higher (Figure 1) than that for the infections of certain protoplasts with TMV, CMV, CCMV, or TNDV (Takebe and Otsuki, 1969; Otsuki and Takebe, 1973; Motoyoshi et al., 1973a; Kubo and Takanami, 1979). However, it is similar to that for CYMV infection of cowpea protoplasts (Rao and Hiruki, 1978) and for PVX infection of tobacco protoplasts (Otsuki et al., 1974). A higher pH value of phosphate buffer was also optimal for infection of tomato protoplasts with TMV (Motoyoshi and Oshima, 1975), and tobacco protoplasts with TRV (Kubo et al., 1975b). The effect of pH on virus infection of protoplasts is interpreted to be due to its influence on interactions between virus and PLO, and between virus or a virus-PLO complex and protoplasts. It seems that at pH 6.3, the virus particles assume optimal negative charges so as to form a positively charged virus-PLO complex for the maximum adsorption to the negatively charged protoplast surface. By virtue of the increased pH, phosphate buffer indirectly improves the stability of the protoplasts, as they are unstable at lower pH values (Takebe, 1975a; Motoyoshi and Oshima, 1975).

The extent of infection of protoplasts also depends on the concentration of virus in the inoculum. In this investigation, percentage of infected protoplasts increased with the increase in CYMV concentration up to 3 μ g per ml (Figure 3). Further increases in the virus concentration did not result in the increased infection efficiency, but the slight reduction. This reduction can be explained by a shift in the net charge of the virus-PLO complex from

positive to negative charge due to increased virus concentrations in the inoculum. Alternatively, it may be due to a need for increase in PLO concentration in the inoculum. However, the effect of increased PLO concentrations was not examined, as the survival rate of the protoplasts declined sharply at the higher PLO concentrations (Figure 2). For the maximum infection of protoplasts, CYMV required an inoculum virus concentration higher than that of some other viruses (Takebe and Otsuki, 1969; Kubo et al., 1975b). It is interesting to note that PVX which belongs to the same potex group as CYMV, also required a high inoculum virus concentration (Otsuki et al., 1974).

The time of incubation of the protoplasts with the inoculum also influenced the percentage of infection. A 10 minute incubation was sufficient for relatively good infection, and there was no considerable difference in the rate of infection with longer incubation times (Figure 4). The exposure time of 10 minutes was also considered sufficient for infection of protoplasts with TMV, CCMV and BMV (Otsuki et al., 1972; Motoyoshi and Oshima, 1975; Motoyoshi et al., 1973a; Okuno et al., 1977), although a five minute exposure of tobacco protoplasts to PVX was enough to give a maximum infection (Otsuki et al., 1974).

One of the important aspects of the study concerning virus multiplication is the role of inclusion bodies produced in infected cells. CYMV is known to produce various kinds of intracellular inclusion bodies (Christie, 1967; Rao et al., 1978). However, very little is known about the sequential biogenesis of these bodies.

The use of protoplasts can greatly help understand the formation and the role of inclusion bodies in vivo. In nature CYMV often occurs in combination with WCMV (Pratt, 1961). With the protoplast system it may be possible to study the interaction between these distantly related viruses, and also between different strains of CYMV.

In its application to practical fields, it must be pointed out that the protoplast system offers a significant possibility to develop CYMV-resistant plants. The protoplasts isolated from plants systemically infected with CYMV or the protoplasts inoculated with CYMV, can regenerate cell walls in vitro (Quraishi and Hiruki, unpublished data). These cells form callus-like colonies and subsequently whole plants can be regenerated. Resistant plants may be selected by repeated challenge inoculations of subsequent plant generations with the virus.

PLATES AND LEGENDS

Plate 1. A light micrograph of protoplasts isolated from pea leaf mesophyll suspended in 0.7 M-mannitol. The scale represents 20 μm .

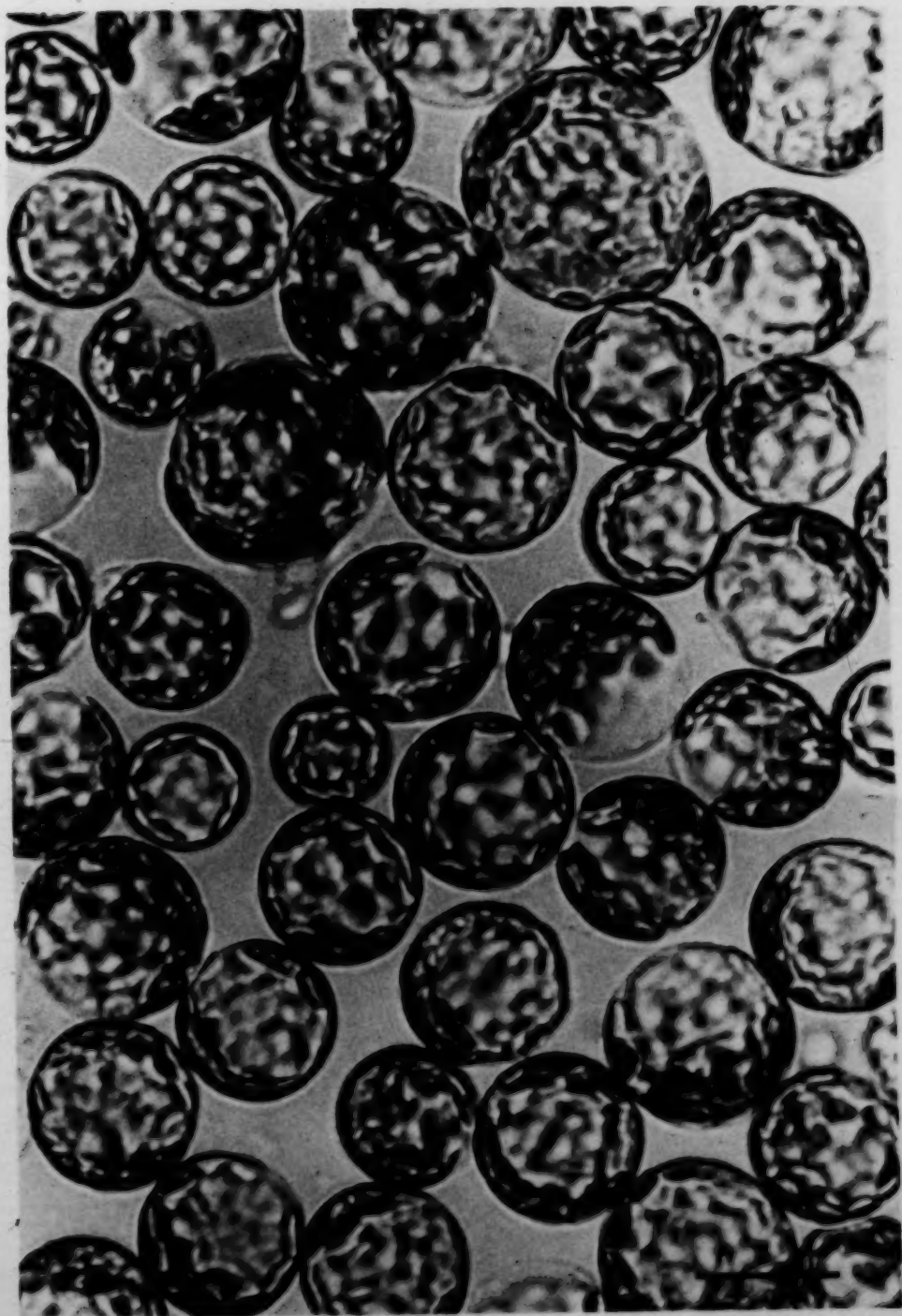
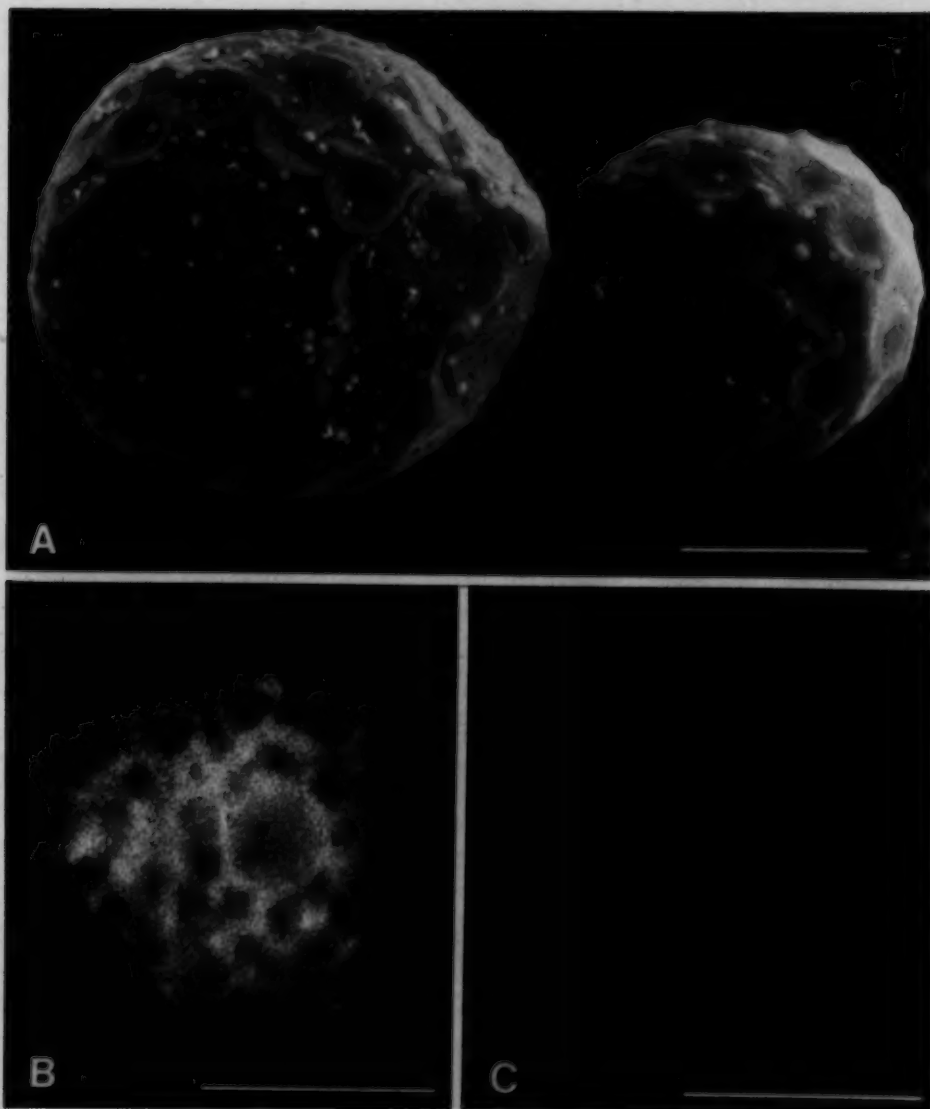


Plate 2.

- A. A scanning electron micrograph of pea mesophyll protoplasts. The scale represents 10 μm .
- B. and C. Fluorescence micrographs of pea leaf mesophyll protoplasts stained with fluorescent antibody to clover yellow mosaic virus (CYMV): B. Inoculated with CYMV. C. Uninoculated. The scales represent 10 μm .



BIBLIOGRAPHY

- AGRAWAL, H., L. BOS, and M. CHESSIN. 1962. Distribution of clover yellow mosaic virus and white clover mosaic viruses on white clover in the United States. *Phytopathology* 52: 517-519.
- ALBLAS, F., and J. F. BOL. 1977. Factors influencing the infection of cowpea mesophyll protoplasts by alfalfa mosaic virus. *J. Gen. Virol.* 36: 175-185.
- ALBLAS, F., and J. F. BOL. 1978. Coat protein is required for infection of cowpea protoplasts with alfalfa mosaic virus. *J. Gen. Virol.* 14: 653-656.
- ANDERSON, T. F. 1951. Techniques for the preservation of three-dimensional structure in preparing specimen for the electron microscope. *Trans. N. Y. Acad. Sci. Ser. 2*, 13: 130-134.
- AOKI, S., and I. TAKEBE. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. *Virology* 39: 439-448.
- BARKER, H., and B. D. HARRISON. 1977. Infection of tobacco mesophyll protoplasts with raspberry ringspot virus alone and together with tobacco rattle virus. *J. Gen. Virol.* 35: 125-133.
- BARKER, H., and B. D. HARRISON. 1978. Double infection interference and superinfection in protoplasts exposed to two strains of raspberry ringspot virus. *J. Gen. Virol.* 40: 647-658.
- BEIER, H., and G. BRUENING. 1975. The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. *Virology* 64: 272-276.
- BEIER, H., D. J. SILVER, M. L. RUSSELL, and G. BRUENING. 1977. Survey of susceptibility of cowpea mosaic protoplasts and intact plants from Vigna sinensis lines. *Phytopathology* 67: 917-921.
- BURGESS, J., F. MOTOYOSHI, and E. N. FLEMING. 1973a. Effects of poly-L-ornithine on isolated tobacco mesophyll protoplasts: Evidence against stimulated pinocytosis. *Planta* 111: 199-208.
- BURGESS, J., F. MOTOYOSHI, and E. N. FLEMING. 1973b. The mechanism of infection of plant protoplasts by viruses. *Planta* 112: 323-332.

- BURGESS, J., F. MOTOYOSHI, and E. N. FLEMING. 1974. Structural changes accompanying infection of tobacco protoplasts with two spherical viruses. *Planta* 117: 133-144.
- CAMPBELL, D. H., J. H. GARVEY, N. E. CREMER, and D. H. SUSSDORF. 1970. Isolation of rabbit antibodies and their subunits. In *Methods in Immunology*, pp. 183-234. W. A. Benjamin Inc., New York.
- CASSELLS, A. C., and M. BARLASS. 1978a. A method for the isolation of stable mesophyll protoplasts from tomato leaves throughout the year under standard conditions. *Physiol. Plant.* 42: 236-242.
- CASSELLS, A. C., and M. BARLASS. 1978b. The infection of TMV initiation in isolated protoplasts by polyethylene glycol. *Virology* 87: 459-462.
- CASSELLS, A. C., and A. A. GATENBY. 1975. The use of lissamine rhodamine B conjugated antibody for the detection of tobacco mosaic virus antigen in tomato mesophyll protoplasts. *Z. Naturforsch* 30c: 696-697.
- CHIN, J. C., and K. J. SCOTT. 1979. A large scale isolation procedure for cereal mesophyll protoplasts. *Ann. Bot.* 43: 23-32.
- CHRISTIE, R. G. 1967. Rapid staining procedures for differentiating plant virus inclusions in epidermal strips. *Virology* 31: 268-271.
- COCKING, E. C. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature (London)* 187: 927.
- COCKING, E. C. 1966. An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. *Planta* 68: 206-214.
- COCKING, E. C. 1970. Virus uptake, cell wall regeneration, and virus multiplication in isolated plant protoplasts. *Int. Rev. Cytol.* 28: 89-124.
- COCKING, E. C., and E. PONJAR. 1969. An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. *J. Gen. Virol.* 4: 305-312.
- COUTTS, R. H. A., E. C. COCKING, and B. KASSANIS. 1972. Infection of tobacco mesophyll protoplasts with tobacco mosaic virus. *J. Gen. Virol.* 17: 289-294.
- DE ZOETEN, G. A., A. M. ASSINK, and A. VAN KAMMEN. 1974. Association of cowpea mosaic virus-induced double-stranded RNA with cytopathological structure in infected cells. *Virology* 59: 341-355.

- FORD, R. E., and J. R. BAGGETT. 1965. Reactions of plant introduction lines of Pisum sativum to alfalfa mosaic, clover yellow mosaic and pea streak viruses, and to powdery mildew. Plant Dis. Repr. 49: 787-789.
- GREGORY, D. W., and E. C. COCKING. 1963. The use of polygalacturonase for the isolation of plant protoplasts and vacuoles. Biochem. J. 88: 40.
- GREGORY, D. W., and E. C. COCKING. 1965. The large scale isolation of protoplasts from immature tomato fruit. J. Cell Biol. 24: 143-146.
- GROUT, B. W. W., and R. H. H. COUTTS. 1974. Additives for enhancement of fusion and endocytosis in higher plant protoplasts: an electrophoretic study. Plant Science Letters 2: 397-403.
- HARRISON, B. D., S. KUBO, D. J. ROBINSON, and A. M. HUTCHESON. 1976. The multiplication cycle of tobacco rattle virus in tobacco mesophyll protoplasts. J. Gen. Virol. 33: 237-248.
- HATTA, T., T. NAKAMOTO, Y. TAKAGI, and R. USHIYAMA. 1971. Cytological abnormalities of mitochondria induced by infection with cucumber green mottle mosaic virus. Virology 45: 292-297.
- HIBI, T., G. REZELMAN, and A. VAN KAMMEN. 1975. Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. Virology 64: 308-318.
- HIBI, T., and K. YORA. 1972. Electron microscopy of tobacco mosaic virus infection in tobacco mesophyll protoplasts. Ann. Phytopathol. Soc. Japan 38: 350-356.
- HIRUKI, C., D. V. RAO, and M. H. CHEN. 1979. A survey report on virus and mycoplasma diseases of forage legume crops in central and northern Alberta. Ann. Meeting of the Alberta Regional Group of the Can. Phytopath. Society.
- HIRUKI, C., P. SHUKLA, and D. V. RAO. 1976. Occurrence of clover yellow mosaic virus aggregates in transfer cells of Pisum sativum. Phytopathology 66: 594-597.
- HONDA, Y., S. KAJITA, C. MATSUI, Y. OTSUKI, and I. TAKEBE. 1975. An ultrastructure study of the infection of tobacco mesophyll protoplasts by potato virus X. Phytopath. Z. 84: 66-74.
- HONDA, Y., C. MATSUI, Y. OTSUKI, and I. TAKEBE. 1974. Ultrastructure of tobacco mesophyll protoplasts inoculated with cucumber mosaic virus. Phytopathology 64: 30-34.

- HOWELL, S. H., and R. HULL. 1978. Replication of cauliflower mosaic virus and transcription of its genome in turnip leaf protoplasts. *Virology* 86: 468-481.
- HUBER, R., G. REZELMAN, T. HIBI, and A. VAN KAMMEN. 1977. Cowpea mosaic virus infection of protoplasts from Samsun tobacco leaves. *J. Gen. Virol.* 34: 315-323.
- HUGHES, B. G., F. G. WHITE, and M. A. SMITH. 1978. Effect of plant growth, isolation and purification conditions on barley protoplast yield. *Biochem. Physiol. Pflanz.* 172: 67-78.
- JENSEN, R. G., R. I. B. FRANCKI, and M. ZAITLIN. 1971. Metabolism of separated leaf cells. I. Preparation of photosynthetically active cells from tobacco. *Plant Physiol.* 48: 9-13.
- KAO, K. N., and M. R. MICHAYLUK. 1974. A method for high-frequency intergeneric fusion of plant protoplasts. *Planta* 115: 355-367.
- KASSANIS, B., and R. F. WHITE. 1974. A simplified method of obtaining tobacco protoplasts for infection with tobacco mosaic virus. *J. Gen. Virol.* 24: 447-452.
- KASSANIS, B., R. F. WHITE, R. H. TURNER, and R. D. WOODS. 1977. The mechanism of virus entry during infection of tobacco protoplasts with TMV. *Phytopath. Z.* 88: 215-228.
- KASSANIS, B., R. F. WHITE, and R. D. WOODS. 1975. Inhibition of multiplication of tobacco mosaic virus in protoplasts by antibiotics and its prevention by divalent metals. *J. Gen. Virol.* 28: 185-191.
- KLERCKER, V. J. 1892. Eine methode zur isolierung lebender protoplasten. *Ofrere Vetensk Akad. Forh. Stockholm* 49: 463-475.
- KOIKE, M., T. HIBI, and K. YORA. 1977. Infection of cowpea mesophyll protoplasts with cucumber mosaic virus. *Virology* 83: 413-416.
- KUBO, S., B. D. HARRISON, and H. BARKER. 1975a. Defined conditions for growth of tobacco plants as source of protoplasts for virus infection. *J. Gen. Virol.* 28: 255-257.
- KUBO, S., B. D. HARRISON, and D. J. ROBINSON. 1974. Effect of phosphate on the infection of tobacco protoplasts by tobacco rattle virus. *Intervirology* 3: 382-387.
- KUBO, S., B. D. HARRISON, D. J. ROBINSON, and M. A. MAYO. 1975b. Tobacco rattle virus in tobacco mesophyll protoplasts: infection and virus multiplication. *J. Gen. Virol.* 27: 293-304.

- KUBO, S., D. J. ROBINSON, B. D. HARRISON, and A. M. HUTCHESON. 1976. Uptake of tobacco rattle virus by tobacco protoplasts, and the effect of phosphate on infection. *J. Gen. Virol.* 30: 287-298.
- KUBO, S., and Y. TAKANAMI. 1979. Infection of tobacco mesophyll protoplasts with tobacco necrotic dwarf virus, a phloem-limited virus. *J. Gen. Virol.* 42: 387-398.
- MAYO, M. A. 1978. The effect of protoplast concentration during inoculation on the infection of tobacco protoplasts. *Inter-virology* 9: 184-188.
- MAYO, M. A., and I. M. ROBERTS. 1978. Inoculation of tobacco protoplasts with tobacco rattle virus: Experiments with aggregates formed in inocula. *J. Gen. Virol.* 40: 19-30.
- MAYO, M. A., and I. M. ROBERTS. 1979. Some effects of buffer on the infectivity and appearance of virus inocula used for tobacco protoplasts. *J. Gen. Virol.* 44: 691-698.
- MEYER, Y. 1974. Isolation and culture of tobacco mesophyll protoplasts using a saline medium. *Protoplasma* 81: 363-372.
- MISAWA, T., and Y. EHARA. 1966. Electron microscopic observation of host cells infected with cucumber mosaic virus. *Tohoku J. Agric. Res.* 16: 159-173.
- MORRIS-KRSINICH, B., R. HULL, and M. RUSSO. 1979. Infection of turnip leaf protoplasts with turnip rosette virus. *J. Gen. Virol.* 43: 339-347.
- MOTOYOSHI, F., J. B. BANCROFT, and J. W. WATTS. 1973a. A direct estimate of the number of cowpea chlorotic mottle virus particles adsorbed by tobacco protoplasts that become infected. *J. Gen. Virol.* 21: 159-161.
- MOTOYOSHI, F., J. B. BANCROFT, and J. W. WATTS. 1974a. The infection of tobacco protoplasts with a variant of brome mosaic virus. *J. Gen. Virol.* 25: 31-36.
- MOTOYOSHI, F., J. B. BANCROFT, J. W. WATTS, and J. BURGESS. 1973b. The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA. *J. Gen. Virol.* 20: 177-193.
- MOTOYOSHI, F., and R. HULL. 1974. The infection of tobacco protoplasts with pea enation mosaic virus. *J. Gen. Virol.* 24: 89-99.
- MOTOYOSHI, F., R. HULL, and I. FLACK. 1975. Infection of tobacco mesophyll protoplasts by alfalfa mosaic virus. *J. Gen. Virol.* 27: 263-266.

- MOTOYOSHI, F., and N. OSHIMA. 1975. Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. *J. Gen. Virol.* 29: 81-91.
- MOTOYOSHI, F., and N. OSHIMA. 1976. The use of tris-HCl buffer for inoculation of tomato protoplasts with tobacco mosaic virus. *J. Gen. Virol.* 32: 311-314.
- MOTOYOSHI, F., J. W. WATTS, and J. B. BANCROFT. 1974b. Factors influencing the infection of tobacco protoplasts by cowpea chlorotic mottle virus. *J. Gen. Virol.* 25: 245-256.
- NAGATA, T., and S. ISHII. 1979. A rapid method for isolation of mesophyll protoplasts. *Can. J. Bot.* 57: 1820-1823.
- NAGATA, T., and G. MELCHERS. 1978. Surface charge of protoplasts and their significance in cell - cell interaction. *Planta* 142: 235-238.
- NISHIMURA, M., and T. AKAZAWA. 1975. Photosynthetic activity of spinach leaf protoplasts. *Plant Physiol.* 55: 712-716.
- OKUNO, T., and I. FURUSAWA. 1977. A simple method for the isolation of intact mesophyll protoplasts from cereal plants. *Plant Cell Physiol.* 18: 1357-1362.
- OKUNO, T., and I. FURUSAWA. 1978a. Modes of infection of barley protoplasts with brome mosaic virus. *J. Gen. Virol.* 38: 409-418.
- OKUNO, T., and I. FURUSAWA. 1978b. The use of osmotic shock for inoculation of barley protoplasts with brome mosaic virus. *J. Gen. Virol.* 39: 187-190.
- OKUNO, T., I. FURUSAWA, and C. HIRUKI. 1977. Infection of barley protoplasts with brome mosaic virus. *Phytopathology* 67: 610-615.
- OTSUKI, Y., and I. TAKEBE. 1969a. Fluorescent antibody staining of tobacco mosaic virus antigen in tobacco mesophyll protoplasts. *Virology* 38: 497-499.
- OTSUKI, Y., and I. TAKEBE. 1969b. Isolation of intact mesophyll cells and their protoplasts from higher plants. *Plant and Cell Physiology* 10: 917-921.
- OTSUKI, Y., and I. TAKEBE. 1973. Infection of tobacco mesophyll protoplasts by cucumber mosaic virus. *Virology* 52: 433-438.

- OTSUKI, Y., and I. TAKEBE. 1976a. Double infection of isolated tobacco leaf protoplasts by two strains of tobacco mosaic virus. In: Biochemistry and cytology of plant parasite interaction (Tomiyama, K., J. M. Daly, I. Uritani, H. Oku, and S. Ouchi, eds.), pp. 213-222, Kodansha, Tokyo.
- OTSUKI, Y., and I. TAKEBE. 1976b. Double infection of isolated tobacco mesophyll protoplasts by unrelated plant viruses. *J. Gen. Virol.* 30: 309-316.
- OTSUKI, Y., I. TAKEBE, Y. HONDA, S. KAJITA, and C. MATSUI. 1974. Infection of tobacco mesophyll protoplasts by potato virus X. *J. Gen. Virol.* 22: 375-385.
- OTSUKI, Y., I. TAKEBE, Y. HONDA, and C. MATSUI. 1972. Ultra-structure of infection of tobacco mesophyll protoplasts by tobacco mosaic virus. *Virology* 49: 188-194.
- PLOWE, J. Q. 1931. Membranes in the plant cell. 1. Morphological membranes at protoplasmic surfaces. *Protoplasma* 12: 196-220.
- POWER, J. B., and E. C. COCKING. 1968. A simple method for the isolation of very large number of leaf protoplasts by using mixtures of cellulase and pectinase. *Proceedings of the Biochemical Society. Biochem. J.* 111: 33.
- POWER, J. B., and E. C. COCKING. 1970. Isolation of leaf protoplasts: Macromolecule uptake and growth substance response. *J. Exp. Bot.* 21: 64-70.
- PRATT, M. J. 1961. Studies on clover yellow mosaic and white clover mosaic viruses. *Can. J. Bot.* 39: 655-665.
- PRATT, M. J. 1967. Reduced winter survival and yield of clover infected with clover yellow mosaic virus. *Can. J. Plant Sci.* 47: 289-294.
- RENAUDIN, J., J. M. BOVE, Y. OTSUKI, and I. TAKEBE. 1975. Infection of *Brassica* leaf protoplasts by turnip yellow mosaic virus. *Mol. Gen. Genet.* 141: 59-68.
- RAO, D. V., and C. HIRUKI. 1978. Infection of cowpea mesophyll protoplasts with clover yellow mosaic virus. *J. Gen. Virol.* 38: 303-312.
- RAO, D. V., C. HIRUKI, and T. MATSUMOTO. 1980. The identification and characterization of a vetch strain of clover yellow mosaic virus. *Phytopath. Z.* 97: (In press).

- RAO, D. V., P. SHUKLA, and C. HIRUKI. 1978. In situ reaction of clover yellow mosaic virus (CYMV) inclusion bodies with fluorescent antibodies to CYMV. *Phytopathology* 68: 1156-1159.
- RUESINK, A. W., and K. V. THIMANN. 1965. Protoplasts from the avena coleoptile. *Proc. Natl. Acad. Sci. U.S.A.* 54: 56-64.
- RUESINK, A. W., and K. V. THIMANN. 1966. Protoplasts: preparation from higher plants. *Science* 154: 280-281.
- SAKAI, F., J. W. WATTS, J. R. O. DAWSON, and J. B. BANCROFT. 1977. Synthesis of proteins in tobacco protoplasts infected with cowpea chlorotic mottle virus. *J. Gen. Virol.* 34: 285-293.
- SARKAR, S. 1977. Use of protoplasts for plant virus studies. In: 'Methods in virology,' Vol. VI (K. Maramorosch and H. Koprowski, eds.), pp. 435-456, Academic Press, New York.
- SCHILDE-RENTSCHLER, L. 1973. A simple method for the preparation of plant protoplasts. *Z. Naturforsch* 27b: 208-209.
- SHALLA, T. A., and L. J. PETERSON. 1973. Infection of isolated plant protoplasts with potato virus X. *Phytopathology* 63: 1125-1130.
- SHALLA, T. A., and J. F. SHEPARD. 1972. The structure and antigenic analysis of amorphous inclusion bodies induced by potato virus X. *Virology* 49: 654-667.
- SHEPARD, J. F. 1975. Regeneration of plants from protoplasts of potato virus X-infected tobacco leaves. *Virology* 66: 492-501.
- SHEPARD, J. F., and R. E. TOTTE. 1975. Isolation and regeneration of tobacco mesophyll cell protoplasts under low osmotic conditions. *Plant Physiol.* 55: 689-694.
- SUGIMURA, Y., and R. USHIYAMA. 1975. Cucumber green mottle mosaic virus infection and its bearing on cytological alterations in tobacco mesophyll protoplasts. *J. Gen. Virol.* 29: 93-98.
- SUZUKI, M., I. TAKEBE, S. KAJITA, Y. HONDA, and C. MATSUI. 1977. Endocytosis of polystyrene spheres by tobacco leaf protoplasts. *Exp. Cell Res.* 105: 127-136.
- TAKEBE, I. 1975a. Protoplasts from leaf mesophyll and their infection by plant viruses. *Rev. Plant Protec. Res.* 8: 136-150.
- TAKEBE, I. 1975b. The use of protoplasts in plant virology. *Ann. Rev. Phytopathol.* 13: 105-125.

- TAKEBE, I. 1977. Protoplasts in the study of plant virus replication. In: 'Comprehensive Virology 11' (H. Fraenkel-Conrat and R. W. Wagner, eds.), pp. 348, Plenum Press, New York and London.
- TAKEBE, I., and Y. OTSUKI. 1969. Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. *Proc. Nat. Acad. Sci. Wash.* 64: 843-848.
- TAKEBE, I., Y. OTSUKI, and S. AOKI. 1968. Isolation of tobacco mesophyll cells in intact and active state. *Plant and Cell Physiol.* 9: 115-124.
- TAKEBE, I., Y. OTSUKI, Y. HONDA, and C. MATSUI. 1975. Penetration of plant viruses into isolated tobacco leaf protoplasts. In: *Proceedings of the first intersectional congress of the International Association of Microbiological Societies*, Vol. 3 (T. Hasegawa, ed.), pp. 55-64. Science Council of Japan, Tokyo.
- VASIL, I. 1976. The progress, problems, and prospects of plant protoplast research. *Advan. Agron.* 28: 121-160.
- WATTS, J. W., and J. M. KING. 1973. A method for the preparation of plant protoplasts. *Z. Naturforsch.* 28c: 231.
- WATTS, J. W., F. MOTOYOSHI, and J. M. KING. 1974. Problems associated with the production of stable protoplasts of cells of tobacco mesophyll. *Ann. Bot.* 38: 667-671.
- WHATLEY, F. R. 1956. Cytochemical methods. In: 'Modern Methods of Plant Analysis,' (K. Peach and M. W. Tracy, eds.) 1: 452-467. Gottingen: Springer.
- WYATT, S. D., and J. G. SHAW. 1975. Retention and dissociation of tobacco mosaic virus by tobacco protoplasts. *Virology* 63: 459-465.
- YARWOOD, C. E. 1952. The phosphate effect in plant virus inoculations. *Phytopathology* 42: 137-143.
- ZAITLIN, M. 1959. Isolation of tobacco leaf cells capable of supporting virus multiplication. *Nature* 184: 1002-1003.
- ZHURAVLEV, Y. N., N. F. PISETSKAYA, L. A. SHUMILOVA, T. I. MUROZOK, and V. G. REIFMAN. 1975. Attachment of labelled TMV to tobacco mesophyll protoplasts. *Virology* 64: 43-48.